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# Assessment of musk and UV filter compounds in wild mussels collected in Portugal coast

**Master in Quality Control**

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**Work performed under the supervision of Doctor Sara Cristina da Silva Cunha**

**and co-supervison of Prof. Doctor José de Oliveira Fernandes**

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# Determinação de fragrâncias sintéticas e filtros UV em mexilhões colhidos na costa portuguesa

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More than a master's thesis, this work appears as the accomplishment of one more step in the path that I decided to go through and that began almost a decade ago. When I graduated, I thought it would be the last step, not happy, I continued to study in an area that seduces me, Quality and my favorite analytical technique, Chromatography. Reconciling work with study and family is a huge challenge, often frustrating and not easy. At this point and looking back I feel a great happiness because it was possible and very gratifying. My life has been like this, twists and turns, parts here and there, the urge to finish and never being finished, trying to do everything in time, but time constantly overtaking me ... In this situation, it helps to meet people who inspire us in different ways and help us walk forward and not give up, once again I feel happy because I have a great collection of them around me!

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"Há um tempo em que é preciso abandonar as roupas usadas, que já tem a forma do nosso corpo, e esquecer os nossos caminhos, que nos levam sempre aos mesmos lugares. É o tempo da travessia: e, se não ousarmos fazê-la, teremos ficado, para sempre, à margem de nós mesmos."

Fernando Pessoa

## ABSTRACT

In the last few years the usage of personal care products (PCPs) such as musk fragrances and UV filters has rapidly increased leading to a rise of deleterious effect in the marine biota. Even at low doses, PCPs may cause synergic toxicity effects and cumulative stress in exposed organisms. As most of musk and UV filter compounds are hydrophobic, they tend to accumulate in aquatic organisms, the main route of human contamination. Facing an increasing consumption of seafood, more studies are needed in order to access the real threat that these compounds represent to aquatic environment and ultimately to humans.

Mussels have been extensively used as “sentinel” species in a large number of monitoring programs because they combine many characteristics required by bioindicator species: abundance and easy to catch, wide geographical distribution and restrict home range.

In view of this, the main objective of the present work was to develop and validate a method based on Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) extraction followed by Dispersive Liquid Liquid Micro Extraction (DLLME) and Gas Chromatography with Mass Spectrometry (GC–MS) analysis for the simultaneous determination of musks and UV filters in wild mussel samples. The optimized method was further used in the assessment of musks and UV filters in mussel samples collected from seven sites along the 1,115 miles of coastline in Portugal during 2015.

Very good figures for linearity (correlation coefficient > 0.995), intra and inter-day repeatability (maximum coefficient of variation of 15%) and recovery (69-72%) were obtained for all seven musks and five UV filters under study. Detection and quantification limits varied between 0.5 to 50 ng/g (dw) for musk fragrances and from 0.5 and 5 ng/g (dw) for UV filters. The application of the method to mussel samples showed the presence of 5 out 7 musks studied, with positive levels ranging from 9.3 to 159.4 ng/g (dw). The same samples showed the presence of all the 5 UV filters studied, with positive concentrations ranging from 5.4 to 622.1 ng/g (dw). The occurrence of the target compounds showed large variations depending on localization or season. Higher levels were detected after the bathing season in October. The highest total concentrations of musk and UV filters were found in samples collected in Algés, with levels up to 1077.4 ng/g (dw). Overall the study showed that musks and UV filters contamination was widespread in mussels along the coastal waters of Portugal. The levels of these contaminants tend to be higher in areas of high population density after the summer period.



**Keywords**: Musk fragrances; UV filters; QuEChERS; seafood.

## RESUMO

Nos últimos anos, a utilização de produtos de higiene pessoal que contêm químicos como fragâncias sintéticas e filtros UV tem aumentado rapidamente. Este aumento na utilização e consequente descarga para o meio aquático provoca efeitos nefastos nos organismos aquáticos. Como a maioria das fragâncias sintéticas e dos filtros UV apresentam um carácter hidrofóbico, tendem a acumular-se nos organismos aquáticos. Perante o aumento do consumo de pescado, esta representará uma via cada vez mais importante de contaminação humana. Mais estudos são necessários para avaliar o verdadeiro impacto que estes compostos têm no meio aquático e, em última instância, nos seres humanos.

Os mexilhões têm sido amplamente utilizados como bioindicadores em programas de monitorização ambiental pois reúnem características ideais, tais como abundância, facilidade de captura, ampla distribuição geográfica e comportamento séssil.

O principal objetivo deste trabalho foi adaptar e validar um método baseado na combinação de metodologias de tratamento e extracção de amostra “QuEChERS” (do inglês Quick, Easy, Cheap, Effective, Rugged and Safe) e microextração dispersiva líquido-líquido (DLLME) seguida da análise por cromatografia gasosa com espectrometria de massa (GC-MS) para a determinação simultânea de fragâncias sintéticas e filtros UV em amostras de mexilhões recolhidas em sete locais ao longo dos 943 km de costa em Portugal, durante 2015.

Na optimização do método, coeficientes de correlação superiores a 0,995 para a linearidade, coeficientes de variação (RSD %) inferiores a 15% para a repetibilidade e reprodutibilidade e valores de recuperação entre 69 e 72% foram obtidos para as sete fragâncias sintéticas e cinco filtros UV em estudo. Os limites de detecção e de quantificação variaram entre 0,5 a 50 ng/g (peso seco) para fragâncias sintéticas e entre 0,5 e 5 ng/g (peso seco) para os filtros UV. As amostras analisadas apresentaram 5 das 7 fragâncias sintéticas em estudo, com concentrações positivas entre 9,3 e 159,4 ng/g (peso seco). Todos os filtros UV foram detectados nas amostras com concentrações positivas entre 5,4 e 622,1 ng/g (peso seco). Verificou-se uma variação sazonal e geográfica na ocorrência destes compostos. Níveis mais elevados foram detectados após a época balnear em Outubro. As maiores concentrações totais de fragâncias sintéticas e filtros UV foram observados em Algés, com níveis até 1077,4 ng/g (peso seco).

No geral, observou-se uma contaminação disseminada de fragâncias sintéticas e filtros UV em mexilhões ao longo das águas costeiras de Portugal. Os níveis destes

contaminantes em mexilhões tendem a ser maiores em áreas de alta densidade populacional após o período de verão. Os mexilhões podem ser um bom bioindicador de fragâncias sintéticas e de filtros UV na avaliação da contaminação das águas costeiras, devido à sua atividade como filtradores.

**Palavras-chave:** fragâncias sintéticas; filtros UV; QuEChERS; pescado.

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## ABBREVIATIONS

**4-MBC** - 3-(4-Methylbenzylidene) camphor

**MeCN –** Acetonitrile

**APCI** - Atmospheric Pressure Chemical Ionisation

**APPI** – Atmospheric Pressure Photoionisation

**BMDBM** - 2-Ethylhexyl 4-methoxycinnamate

**BP-3** - 2-Hydroxy-4-methoxybenzophenone

**BP-4** - 2-Hydroxy-4-methoxy benzophenone-5-sulfonic acid

**BP-5** - 2-Hydroxy-4-methoxy benzophenone-5-sodium sulfonate

**BSTFA** - N,O-bis(trimethylsilyl)trifluoroacetamide

**DCM** – Dichloromethane

**DLLME** – Dispersive Liquid Liquid Micro Extraction

**dSBSE** - Dispersive Stir Bar Solid Extraction

**EI** – Electro Impact ionisation

**ESI** – Eletrospray Ionisation

**EQS** - Environmental quality standards

**EtOH** – Ethanol

**FAO** – Food and Agriculture Organization

**GC-MS** – Gas chromatography Mass Spectrometry

**GC-MS/MS** - Gas chromatography Tandem Mass Spectrometry

**GPC** – Gel Permeation Chromatography

**HPLC –** High Performance Liquid Chromatography

**HRMS** - High resolution MS

**LC-MS -** Liquid chromatography Mass Spectrometry

**LD** - Liquid Desorption

**LLE** – Liquid Liquid Extraction

**LOD** – Limit of Detection

**Log $K_{ow}$ –**Octanol water partition coefficient

**LOQ** – Limit of Quantification

**MAC** - Maximum allowable concentrations

**MAE** – Microwave Assisted Extraction

**MALLE** – Membrane Assisted Liquid Liquid Extraction

**MEPS** - Microextraction using Packed Sorbents

**MetOH** – Methanol

**MNPs** - Magnetic nanoparticles

**MSTFA** - N-methyl-N-(trimethylsilyl) trifluoroacetamide

**OC** – Octocrylene

**PCPs** - Personal Care Products

**PDMS** – Polydimethylsiloxane

**PDMS/DVB** - Polydimethylsiloxane/ divinylbenzene

**PLE** – Pressurized Liquid Extraction

**PMALE** - Polymeric membranes Assisted Liquid Extraction

**POCIS** – Polar Organic Chemical Integrative sampler

**PSA** - Primary-secondary amine

**QuEChERS** - Quick, easy, cheap, effective, rugged and safe

**RP** – Reversed Phase

**SBDE** – Stir Bar Dispersive Extraction

**SIM** – Selected Ion Monitoring

**SLE** – Solid Liquid Extraction

**SPE** – Solid Phase Extraction

**SPF** – Sun Protection Factor

**SPMDs** – Semi Permeable Membrane Devices

**SPME** – Solid Phase Micro Extraction

**TD** –Thermal Desorption

**TOC** – Total Organic Carbon

**UALLE** - Ultrasonic Assisted Liquid Liquid Extraction

**UPLC** - Ultra Performance liquid chromatography

**UV filters** - Ultra violet filters

**WWTPs** - Waste Water Treatment Plants

# CHAPTER 1

## INTRODUCTION

# Chapter 1 – Introduction

## 1.1. Seafood Consumption: a pathway to human contamination

The intake of contaminated food may cause some concerning hazardous effects in human beings (2). Humans are consumers of fish and seafood, being a potential route of human exposure to chemicals in the environment (3). Aquatic organisms store chemical substances either directly from the surrounding environment or from their diet. There are two phenomena associated with this: bioaccumulation, which can be defined as the total uptake of a substance from the environment and its accumulation over time, and biomagnification, in which a substance present in the environment is transferred to the food chain, from organism to organism, being the concentration of that substance in an organism higher to that in the food source (4).

### 1.1.1. Worldwide seafood consumption/production

Seafood consumption plays a crucial role in human health and wellbeing. Fisheries are likely to become even more important as populations continue to increase and the pressures on scarce land for agriculture continue to grow, pushing more people towards fisheries as a 'last-resort' activity. In a world with an increasing demand for available protein, fish and fishery products represent a valuable source, as a portion of 150 g of fish provides about 50–60% of the daily protein requirements for an adult (5).

As it can be seen in Table 1, global fishery capture in marine waters, showed a decrease between 2007 and 2010, from 80.7 to 77.8 million tonnes (5). In 2011, an inversion of the tendency was observed with an increase to 82.6 million tonnes, followed by an estimated new decrease in 2012 to 79.7 million tonnes. Global inland water capture production will reach 11.6 million tonnes in 2012, reflecting an increasing tendency, but its share in total global capture production still does not exceed 13% (6).

Global aquaculture production will attained an all-time high of 66.6 million tonnes in 2012. China alone is estimated to produce 43.5 million tonnes of food fish and being the largest exporter of fish and fishery products. However, since 2011, it has become the world's third-largest importing country, after the United States of America and Japan. The European Union is the largest market for imported fish and fishery products, and its dependence on imports is growing (5).

**Table 1 -** World fisheries and aquaculture production and utilization, adapted from FAO (2014).

| | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 |
|---|---|---|---|---|---|---|
| Production | | | (million tones) | | | |
| **Capture** | | | | | | |
| Inland | 10.1 | 10.3 | 10.5 | 11.3 | 11.1 | 11.6 |
| Marine | 80.7 | 79.9 | 79.6 | 77.8 | 82.6 | 79.7 |
| **Total capture** | **90.8** | **90.1** | **90.1** | **89.1** | **93.7** | **91.3** |
| **Aquaculture** | | | | | | |
| Inland | 29.9 | 32.4 | 34.3 | 36.8 | 38.7 | 41.9 |
| Marine | 20.0 | 20.5 | 21.4 | 22.3 | 23.3 | 24.7 |
| **Total aquaculture** | **49.9** | **52.9** | **55.7** | **59.0** | **62.0** | **66.6** |
| **Total World Fisheries** | **140.7** | **143.1** | **145.8** | **148.1** | **155.7** | **158.0** |
| Utilization | | | | | | |
| For human consumption | 117.3 | 120.9 | 123.7 | 128.2 | 131.2 | 136.2 |
| For other purposes | 23.4 | 22.2 | 22.1 | 19.9 | 24.5 | 21.7 |
| Population (billions) | 6.7 | 6.8 | 6.8 | 6.9 | 7 | 7.1 |
| Per capita food fish suply | 17.6 | 17.9 | 18.1 | 18.5 | 18.7 | 19.2 |

*Note:* Excluding aquatic plants. Totals may not match due to rounding.
1 Data in this section for 2012 are provisional estimates.

The proportion of fisheries production used for direct human consumption increased from about 71 % in the 1980s to more than 86 % (136.2 million tonnes) in 2012, with the remainder (21.7 million tonnes) destined to non-food uses (e.g. fishmeal and fish oil). Per capita food fish supply continued to rise up reaching an estimate of 19.2 million tonnes in 2012 (6). These raising tendencies in fish and seafood consumption, illustrates the potential route of human exposure to chemicals in the environment through diet.

### 1.1.2. Portugal in the EU top of seafood consumption

Portugal, with its Exclusive Economic Zone (EEZ), has an enormous fishing area of approximately 1 700 000 km², the fourth largest in the EU (after France, the UK and Denmark) and the 21$^{st}$ largest worldwide. It has also an extense mainland continental coastline of 942 km long and two large island regions (Madeira and Açores). The country has always relied on fishing as a major mean of subsistence, in particular for the coastal communities that have a great dependence on fisheries and related activities (7).

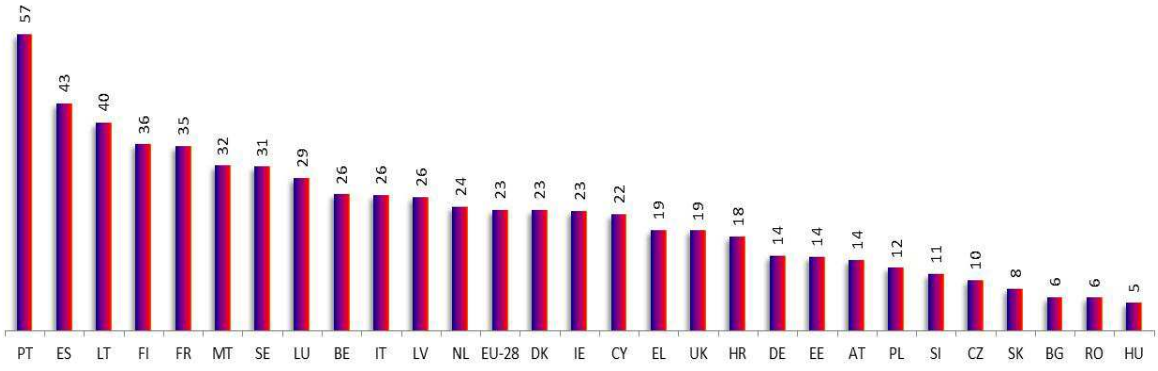



**Fig. 1 –** 2010 data for consumption of fishery and aquaculture products (quantity in live weight (Kg/inhabitant/year) in EU, adapted from Common Fisheries Policy (2014).

Portugal is the largest consumer of fish in the EU, with about 60 kg per capita, per year, well above the Community average of 23 kg per capita/year (Fig. 1). Consumption per capita represents the total apparent consumption divided by the number of inhabitants of a country (6).

Cod is the top preferred specie in Portugal, with an estimated consumption around 30 kg per year (fresh fish equivalent). In terms of catches, sardine is the main Portuguese catch and the leading resource in Portuguese waters (7). In 2007, landings of fresh and chilled fish amounted to 210 000 tonnes, corresponding to a net worth of 275 million € (8).

Among seafood, crustaceans and bivalve molluscs are greatly appreciated by European consumers from a social and gastronomical point of view, particularly in the Mediterranean countries. Seafood and bivalves in Portugal, shellfish in Greece, and oysters, mussels and seafood in France are among of the consumer preferences in those countries. In Portugal, the most consumed crustacean groups are shrimps, prawns and brown crab. Concerning bivalves the most important species consumed are the pullet carpet shell clam, grooved shell dam, representing 66% of the overall bivalve consumption. Mussels and oysters account for 32%. The consumption of shellfish varies seasonally, increasing in summer, particularly for bivalves (9).

This seasonal behaviour in bivalve consumption, if correlated with the reported seasonal occurrence of emerging contaminants in marine environment due to increased human recreational activities (10), can pose a risk to human health. High levels of contaminants were reported by Picot Groz *et al*. (2014) (11) in mussels collected along the Portuguese coast, during summer period, enhancing the need to understand the real impact of these compounds in the aquatic environment and ultimately to humans.

### 1.2. Personal care products as emerging environmental contaminants

Personal Care Products (PCPs) is a generic name that describes a group of chemicals included in different products widely used in daily human life, such as toothpaste, shampoo, cosmetics and even in foods (12). They are not used for treatment of disease, but some may be intended to prevent diseases, as the case of sunscreen agents, protecting against the effects of sun radiation (13).

The terms “personal care products” and “cosmetics” can be used with the same meaning, because there is no apparent difference between the two terms according to the definition, although in everyday language it could refer to slightly different issues. In Article 1 of the European Union (EU) Cosmetics Directive 76/768/EC (14) cosmetics are defined as “any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odours”.

Many chemicals that have not historically been considered as contaminants are present in the environment on a global scale. These “emerging” contaminants, previously unknown chemical pollutants in the environment, and the risks associated with them have been considered a major concern for environmental scientists, due to their frequent detection in the aquatic environment (13).

PCPs are among the most commonly detected compounds in surface water throughout the world, but relatively little is known about their toxicity (13). They are released into the environment unaltered through normal human usage, at levels greater than pharmaceuticals so attention is being given to identify environmental concentrations and potential toxicity (15). The occurrence of PCPs in waters is not a new phenomenon, it has only become more widely evident in the last decade because continually improving of the analytical methodologies that allow even lower detection limits and the ability to detect polar compounds (16). While already used in vast quantities, the consumption and usage of PCPs are only expected to increase. An inevitable consequence of this increased consumption of PCPs is higher levels of their discharge into the environment (16).

Personal care products, such as musk fragrances and sunscreen agents, tend to be very lipophilic, displaying all the qualities of conventional persistent organic pollutants (POPs), ubiquitous, persistent, and bioaccumulative**,** like musk and sunscreen agents (13).

The occurrence of PCPs in the environment highlights the immediate connection between the individual activities of consumers and their environment. In contrast to other types of

pollutants, PCPs owe their origins in the environment directly to their worldwide usage and direct disposal by individuals (17).

PCPs can enter the environment directly, following their application by the user in day by day activities, or indirectly via excretion after some dermal absorption, and through industrial wastewater discharges in their manufacturing processes (1). Disposal of unused or expired PCPs in landfills and to domestic sewage are additional routes to the environment (Fig. 2). The aquatic environment serves as the ultimate receptacle for most PCPs (17).

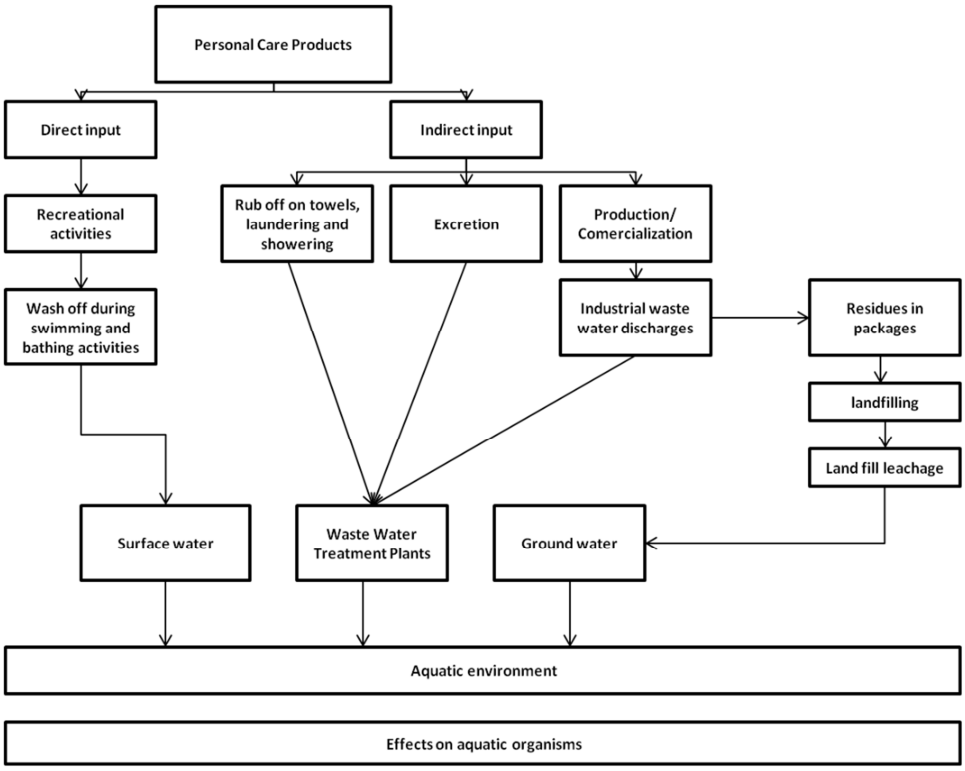



**Fig. 2 -** Sources of Personal Care Products in the aquatic environment, adapted from Giokas *et al*. (2007) (1).

Any chemical introduced to the aquatic domain can lead to continual exposure for aquatic organisms acting as if they were persistent, because their continual infusion into the aquatic environment leads to lifelong multi-generational exposures for aquatic organisms, even if their half-lives are short, reaching a scale of worldwide ubiquity (13).

Although environmental persistence usually is a major determinant of exposure in the environment, for pollutants that are used on a continual basis and are introduced to the environment through Waste Water Treatment Plants (WWTPs), the supply is continually replenished. This is especially true for aquatic organisms, which are captives of their environment and perpetually exposed (13).

The main categories of PCPs, according to their purpose, include disinfectants/antiseptics (e.g. triclosan), preservatives (e.g. parabens), fragrances (e.g. musks), insect repellants (e.g. N, N-diethyl-meta-toluamide), and UV filters (e.g. 4-methylbenzylidene camphor) (18).

Taking in consideration their tendency to bioaccumulate in aquatic biota, this study will focus in two of these categories: musk fragrances and UV filters.

### 1.2.1. Musk fragrances

Fragrances are a group of compounds whose function is to offer a pleasant scent to any manufactured good, being widely used in PCPs. They can be divided into natural fragrances, consisted in floral and animal extracts, and synthetic fragrances or musks.

Probably the most studied class of PCPs, fragrances have been identified in environmental samples over more than 30 years ago by Yamagishi *et al*. (1981) and are believed to be ubiquitous contaminants in the environment (18).

Synthetic musks are the most commonly use fragrances nowadays, which are found in a diversity of products like household chemicals, soaps and detergents, with high concentrations in perfumes, body lotions and deodorants (12). Synthetic musks can be divided into two major classes: the nitro musks, introduced into commerce in late 1800s, phased out of use in many parts of the world because toxicity concerns; and the polycyclic musks. They are both water soluble, but with high octanol/water coefficients indicating potential for bioaccumulation in aquatic species (13).

Nitro musks are being withdrawn from the European market, because of the findings about the toxicity of their transformation products in the environment (transformation of the nitro groups into aniline compounds) (12). As an alternative, polycyclic musks were developed and introduced in market in 1950s. Galaxolide (HHCB) and tonalide (AHTN) are the most used in PCPs and due to their high lipophilicity, they tend to bioaccumulate, affecting the biota at low trophic levels (19). The risk of dermal application in humans is unknown, but their continual exposure through perfumed products is high, the probable continual introduction of these benzoates into sewage treatment systems and directly to recreational waters from the skin leads to the question of risk to aquatic organisms (13).

### 1.2.2. UV filters

Recognition of the harmful effects of ultraviolet (UV) radiation on the skin has triggered the development of chemicals, commonly referred as UV filters, that can absorb UV radiation and attenuate the negative effects of sunlight exposure (1).

UV filters are compounds designed mainly to protect our skin against damage by UV radiation, ideally providing protection in both UVB and UVA range (20). These compounds can either be organic (chemical) absorbers or inorganic (physical) blockers, depending on the basis of their mechanism of action (21).

The focus of this work will be in the organic UV filters (UV filters) because they constitute an enormous group of emerging environmental pollutants, potentially hazardous compounds that have been receiving steadily growing attention over the last decade. They include different families of compounds like camphors, benzophenones, cinnamates, triazines, among others (21).

These chemicals can be found not only in cosmetics but also in other PCPs, including skin care, facial makeup, and lip care products (15) and in other kind of products like food packaging, pharmaceuticals, plastics, textiles, and vehicle maintenance products to prevent photodegradation of polymers and pigments (22).

### 1.3. Legislation regulating marketed products

The three main regulatory systems on cosmetics products over the world are the European Union (EU) Cosmetics Directive, the United States (US) Food and Drug Administration (FDA) and the legislation in Japan (1).

Everyday cosmetics range from products such as soap, shampoo, deodorant, and toothpaste to luxury beauty items including perfumes and makeup. These products are regulated at European level to ensure consumer safety and to secure an internal market for cosmetics (23). The new EU Regulation 1223/2009 has been in force since 11 July 2013, replacing Directive 76/768/EC, which was adopted in 1976 and had been substantially revised on numerous occasions (24).

The European Parliament has recently recognized musk fragrances and UV filters as important organic contaminants of the aquatic environment due to their lipophilic properties (log $K_{ow}$ ≥ 4.30) and their potential for bioaccumulation (23). Emission limit values (ELVs) have not yet been established for this group of substances. However, maximum allowable concentrations (MACs) and annual averages (AAs) for priority substances and other contaminants in surface water and biota are already defined by the Directive 2000/60/EC, such as the case of hexachlorobenzene, hexacyclohexane and mercury, in biota (10, 50 and 20 µg/kg dw, respectively) (11).

*Musk fragrances*

The EU Cosmetics Regulation (24) establishes a set of rules to be fulfilled by every cosmetic product to be placed on the market as well as provides a list of amount of

chemicals allowed in their composition. For example, the musk ketone is allowed in all cosmetic products with the exception of oral products, being the maximum amounts allowed of 1.4% in fine fragrance, 0.56% in eau de toilette and 0.04% in other products.
Due to toxicological aspects such persistence and bioaccumulation tendency, the nitro musk moskene is not allowed to use in Europe being listed in the Annex II of the EU Regulation (EC) N°, 1223/2009 for prohibited substa nces in cosmetics. Musks xylene and ketone were recommended for authorization under REACH and voluntarily banned by the International Fragrance Association (IFRA) (25), being listed in the Annex III list of substances which cosmetic products must not contain except subject to the restrictions laid down. In contrary, the polycyclic musks galaxolide (HHCB) and tonalide (AHTN) are used in high quantities. Due to their lipophilicity and persistence these substances tend to accumulate in fatty tissues causing magnification in the food chain being found in water and aquatic organisms as well as in human fat, blood and breast milk (26).

*UV filters*

Over the past few decades, sun protection measures have been rising as a result of worldwide educational interventions to reduce UV radiation exposure preventing skin damage, premature skin aging, and the risk of developing skin cancer. Consequently, consumers also demand PCPs with better protection against UV radiation (27).
Although they should protect the consumer against adverse effects of the solar radiation, UV filters-containing PCPs have possible adverse effects in humans and in the aquatic environment.
As illustrated in Figure 3, authorized contents of organic UV filters and product formulations of PCPs vary according to legislation in force in the countries/regions of manufacture, resulting in the spatial variation in the occurrence of these chemicals (28).
Many new UV filters have been introduced in the last decade, with improved efficacy and safety. They are not available, however, in some countries, such as the USA, for regulatory reasons. For example, 26 UV filters are accepted by the Therapeutic Goods Administration in Australia and 31 UV filters are allowed in Japan. In Europe, Annex VI of the Regulation 1223/2009 lists 26 organic UV filters, whereas the sunscreen monograph in the USA mentions only 16, including only 10 in common with the EU.

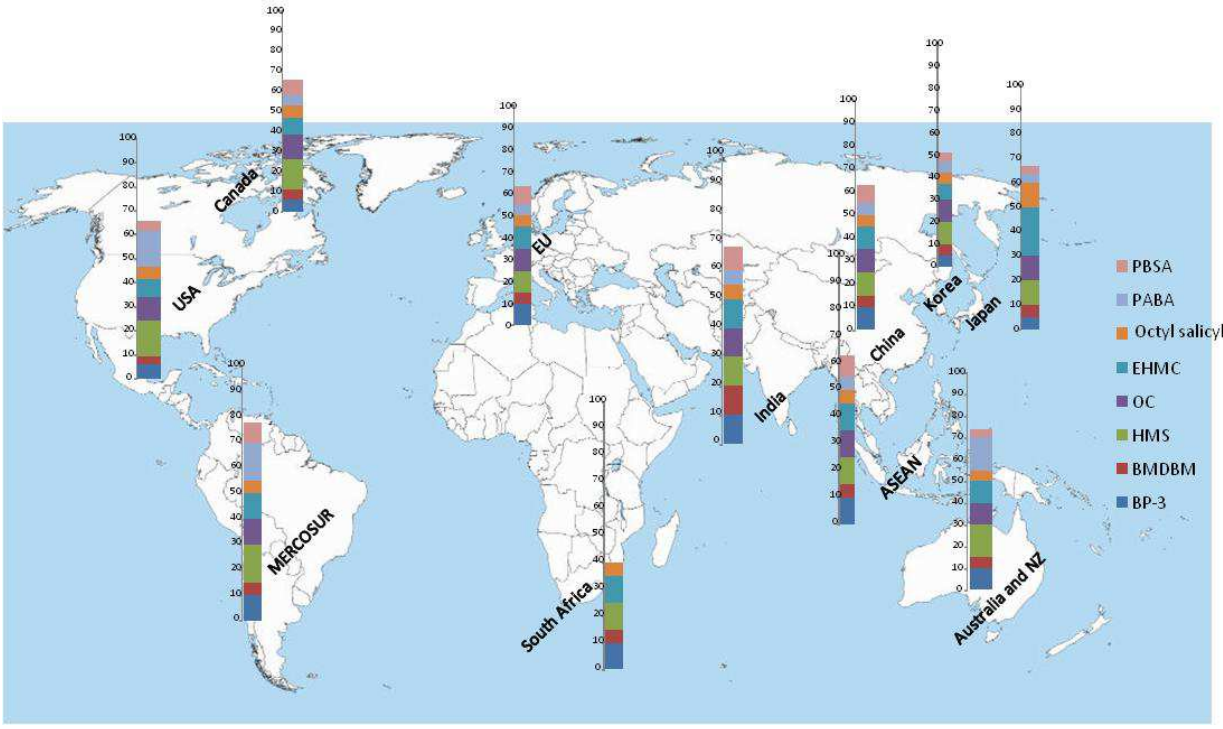



**Fig. 3 –** Geographical variation in the legislation regulating UV filters in marketed products worldwide.

In the Council Directive 98/83/EC (14), no references to UV filters are make on the quality of water intended for human consumption. The European regulation on classification, labelling and packaging (CLP) of substances and mixtures does not imply UV filters. However, if it was used, 12 of the 26 individual UV filters approved for use in cosmetics would meet the CLP classification as 'hazardous to the aquatic environment'. Of these 12 compounds, 2-Hydroxy-4-methoxybenzophenone (BP-3), 2-Ethylhexyl 4-methoxycinnamate (EHMC) and 3-(4-Methylbenzylidene) camphor (4-MBC) would be classified according to the highest toxicity category, and the others would not be classified for lack of information (29). Safety assessment procedures before registration of UV filters are necessary in the United States, Australia, and Japan (2).

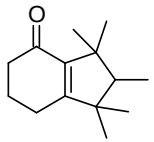
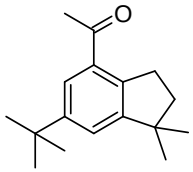
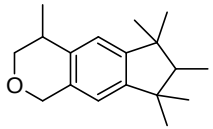
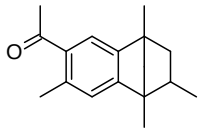
## 1.4. Chemical and physical properties

### 1.4.1. Musk fragrances

The environmental behaviour of a substance is determined by their physical and chemical properties. These include the solubility in water, vapour pressure and the octanol/water partition coefficient (Log $K_{ow}$) or other partition coefficients such as those between water and environmental matrices like soil or the organic material in sewage sludge. Polycyclic musks are substituted indanes and tetralins. Galaxolide and tonalide are the two largest volume products in this group, representing about 95% of the EU market and 90% of the US market for all polycyclic musks. Other members of this group are celestolide (ADBI) and cashmeran (DPMI).

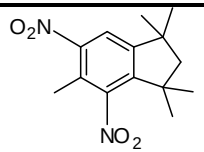
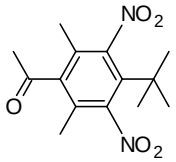
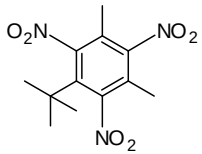
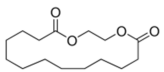
Empirical and estimated values for the physical chemical properties of the polycyclic and nitro musks are included in Table 2 and 3, respectively. Various properties were estimated by quantitative structure–activity relationship (QSARs) models, based on molecular fragments. These estimates may be improved if empirical data for closely related substances are introduced. For the estimation of Log $K_{ow}$, the data for galaxolide were used as additional input to estimate these properties for the others musks. This affects not only the results of Log $K_{ow}$, but also of the estimates for the solubility in water. Common characteristics of the polycyclic musks are the hydrophobic behaviour and poor water solubility. Therefore the substances are expected to sorb onto organic matter and lipids in aquatic organisms.

**Table 2** – Chemical and physical properties of polycyclic musk fragrances.

| Chemical family | Acronym | INCI name | CAS number | Chemical Structure | MW | Log $K_{ow}$ | Solubility in water at 25 °C (mg/L) | Vapor pressure (mm Hg) |
|---|---|---|---|---|---|---|---|---|
| Polycyclic Musks | Cashmeran | 6,7-dihydro-1,1,2,3,3-pentamethyl-4(5H)-indanone (DPMI) | 33704-61-9 |  | 206.32 | 4.9 | 0.17 | $3.9x10^{-2}$ |
| | Celestolide | 4-acetyl-1,1dimethyl-6-*tert*-butylindane (ADBI) | 13171-00-1 |  | 244.38 | 5.93 | 0.22 | $1.5x10^{-4}$ |
| | Galaxolide | 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl cyclopenta-(g)-2-benzopyran (HHCB) | 1222-05-5 |  | 258.40 | 5.9 | 1.75 | $5.2x10^{-4}$ |
| | Tonalide | 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene (AHTN) | 1506-02-1 |  | 258.40 | 5.7 | 1.25 | $4.5x10^{-4}$ |

(-) not mentioned.

**Table 3** – Chemical and physical properties of nitro and macrocyclic musk fragrances.

| Chemical family | Acronym | INCI name | CAS number | Chemical Structure | MW | Log $K_{ow}$ | Solubility in water at 25 °C (mg/L) | Vapor pressure (mm Hg) |
|---|---|---|---|---|---|---|---|---|
| Nitro Musks | Musk moskene | 1,1,3,3,5-pentamethyl-4,6-dinitoindane (MM) | 116-66-5 |  | 280.32 | 5.39 | 0.57 | $8.5 \times 10^{-5}$ |
| | Musk ketone | 4-aceto-3,5-dimethyl-2,6-dinitro-tertbutylbenzene (MK) | 81-14-1 |  | 294.30 | 4.31 | 0.46 | $5.84 \times 10^{-7}$ |
| | Musk xylene | 2,4,6-trinitro-1,3-dimethyl-5-*tert*-butylbenzene (MX) | 81-15-2 |  | 297.26 | 4.9 | 0.15 | $2.25 \times 10^{-7}$ |
| Macrocyclic musk | Ethylene brassylate | 1,4-Dioxacycloheptadecane-5,17-dione | 105-95-3 |  | 270.36 | 4.70 | 1.72 | $3.285 \times 10^{-4}$ |

(-) not mentioned.

### 1.4.2. UV filters

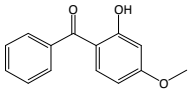
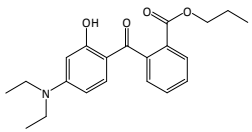
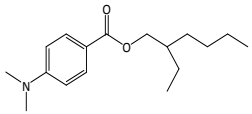
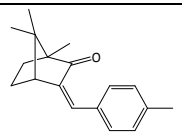
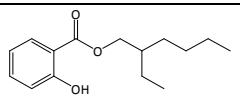
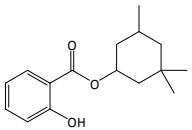
There are two basic types of UV filters, organic and inorganic compounds. The organic UV filters work by absorbing UV light, the inorganic ($TiO_2$, ZnO the only two approved for use in PCPs) also by reflecting and scattering of UV light. Generally, both types of UV filters give good protection against UVB (280-315 nm), and some also offer protection against UVA (315-400 nm) radiation (30).

Table 4 describes relevant chemical and physical properties of some of the UV filters allowed to be used in PCPs in Europe. They represent seven chemical families (31): benzophenone derivatives (two benzene rings joined by a carbonyl group), *p*-aminobenzoic acid derivatives (one benzene ring substituted with an amino group and a carboxyl group in the para position), camphor derivatives (organic compounds classified as terpenoids), salicylate derivatives (containing a monohydroxybenzoic acid group), crylene derivatives (aromatic acrylates) and dibenzoyl methane derivatives (aromatic 1,3-diketone derivative of acetylacetone, where both methyl groups have been substituted by phenyl groups).

A common feature of these compounds is the presence of an aromatic moiety with a side chain, showing different degrees of unsaturation (4). As can be seen in Table 4, some of these compounds are chiral (e.g. EHMC, OC and 4-MBC), but their enantiomers are expected to show the same physicochemical properties (Bester, 2007).

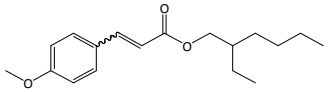
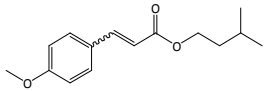
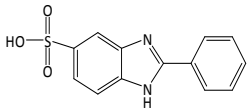
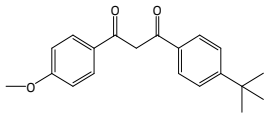
Among the benzophenone derivatives, BP-5 is the salt of BP-4, and both are allowed in cosmetics in a maximum concentration of 5% (w/w) (24).

**Table 4** – Chemical and physical properties of EU approved UV filters in cosmetics.

| Chemical family | Acronym | INCI name | CAS number | Chemical Structure | MW | Log $K_{ow}$ | Solubility in water at 25 °C (mg/L) | pKa |
|---|---|---|---|---|---|---|---|---|
| Benzophenone derivatives | BP-3 | 2-Hydroxy-4-methoxybenzophenone (oxybenzone)Eusolex 4360 | 131-57-7 |  | 228.2 | 3.79; 5.92 | 68.56 | -0.87 |
| | DHHB | diethylamino hydroxybenzoyl hexyl benzoate | 302776-68-7 |  | 397.5 | 6.54 | 8.19x10-3 | - |
| *p*-Aminobenzoic acids derivatives | OD-PABA | octhyl dimethyl p-aminobenzoic acid | 21245-02-3 |  | 277.4 | 5.77 | 0.0053 | 2.39 |
| Camphor derivatives | 4-MBC | 3-(4-Methylbenzylidene)camphor Eusolex 6300 | 38102-62-4 |  | 254.37 | 5.47 5.92 | 0.57 | 7.56 |
| Salicylate derivatives | EHS | 2-ethylhexyl salicylate | 118-60-5 |  | 250.3 | 6 | 0.7 | 8.13 |
| | HS | 3,3,5-trimethylciclohexyl salicylate (homosalate) | 118-56-9 |  | 262.4 | 6.16 | 4.20x10-1 | 8.09 |

(-) not mentioned.

**Table 4** – Chemical and physical properties of EU approved UV filters in cosmetics (cont.).

| Chemical family | Acronym | INCI name | CAS number | Chemical Structure | MW | Log $K_{ow}$ | Solubility in water at 25 °C (mg/L) | pKa |
|---|---|---|---|---|---|---|---|---|
| Cinnamates derivatives | EHMC | 2-Ethylhexyl 4-methoxycinnamate Eusolex 2292 | 103597-45-1 |  | 290.4 | 5.8 | 0.155 | 4.1 |
| | IMC | Isoamyl 4-methoxycinnamate | 71617-10-2 |  | 248.3 | 5.8 | 4.86 | - |
| Crylene derivatives | OC | Octocrylene (Eusolex OCR) | 6197-30-4 |  | 361.49 | 6.88 | 0.004 | - |
| Dibenzoyl methane derivative | BMDBM | 2-Ethylhexyl 4-methoxycinnamate Eusolex 2292 | 70356-09-1 |  | 310.4 | 4.51 Rodil.2008 | 1.52 | 9.74 |

(-) not mentioned.

The octanol-water partition coefficient (log $K_{ow}$) is an indicator of the environmental fate of the chemicals, translating how the compounds are distributed between octanol (which represents the lipids or fats in biota) and water (the aqueous phase). Water solubility provides information on the likely distribution of the chemicals between the different environmental compartments, specially sediment and water and consequently, the potential for environmental or human exposure through release to the aquatic compartment or bioaccumulation in biota. Compounds with high solubility in water and low log $K_{ow}$, are very likely to be found in water. On the other hand, compounds with low solubility in water and high log $K_{ow}$ are more likely to be detected in sediments and biota.

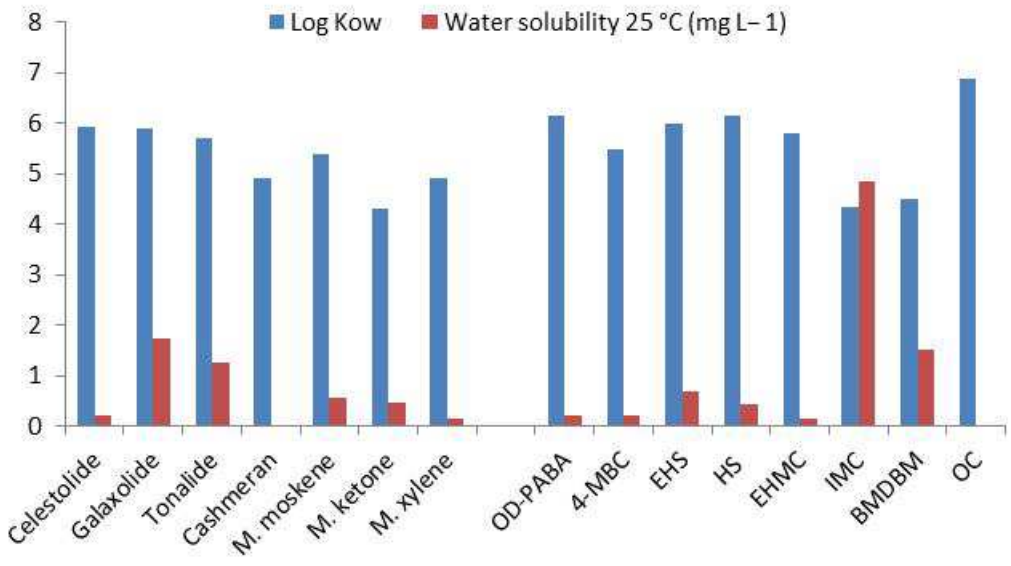



**Fig. 4 –** Log $K_{ow}$ and water solubility values for relevant musk fragrances and UV filters.

Figure 4 illustrates the potential for bioaccumulation of fragrances and UV filters compounds, with high log $K_{ow}$ values (>4.3) and moderately (Galaxolide, Tonalide) to low solubility in water (celestolide, nitro musks, 4-MBC and EHMC). IMC and BMDBM are the most soluble UV filters, but with high log $K_{ow}$.

### 1.5. Occurrence across aquatic environments and biota

Although these organic pollutants may be present at trace levels, their adverse effects on aquatic life, on animals and even on humans are increasing the concern of society and the scientific community. Musk fragrances and UV filters are considered contaminants of emerging concerns because of their intensive use, which in the last years has led to their widespread presence in the environment. Contamination of the aquatic environment with

PCPs may occur through two principal input pathways, “direct inputs” from recreational activities and “indirect inputs” via wastewater treatment plants (WWTPs)(10).

Direct release of UV filters into the aquatic environment from bathing and swimming activities has been summarized in Table 5, being reported as a major environmental source of these chemicals (10). It is likely that release to the environment may occur in beachfront and near-shore ecosystems, as these areas often support extensive recreational activities. In other hand, wastewater containing high levels of PCPs is discharged into the rivers and directly to the sea (32).

**Table 5** – Reported maximum concentrations of most common UV filters in marine environment.

| Sea water (max. conc. ng/L) | | | | | | |
|---|---|---|---|---|---|---|
| Sampling location | UV filter | | | | | Ref. |
| | 4-MBC | BP-3 | EHMC | OC | BM-DBM | |
| Spain | 169 | 603 | 691 | 406 | - | (33) |
| Canary Islands | **1043** | **3316** | **1324** | **756** | **1770** | (34) |
| Japan | nd | - | **1080** | - | - | (35) |
| US coast | - | **2203** | 438 | **3730** | 1298 | (32) |
| Spain | 220 | 308 | 260 | 317 | - | (36) |
| Hong Kong | 379 | **5429** | **4043** | **6812** | 721 | (37) |
| Tokyo | nd | 86 | 95 | 108 | 104 | |
| New York | nd | 178 | 150 | 128 | 87 | |
| Los Angeles | nd | 601 | 138 | 377 | 109 | |
| Shantou | nd | 188 | 78 | 107 | 100 | |
| Chaozhou | nd | 49 | 79 | 102 | nd | |
| Artic | nd | 33 | 66 | 31 | 70 | |
| Slovenia | - | 380 | - | - | - | (38) |
| Spain | 84.6 | 68.6 | 52.5 | - | - | (39) |
| Japan | nd | **1340** | 143 | 78 | nd | (40) |
| Japan (reef) | - | 3.8 | 2.3 | 8.1 | - | |
| Spain | 109.6 | 314.8 | nd | nd | nd | (41) |
| Italy | - | 13 | - | 32 | - | (42) |
| Spain | 358 | 254 | 409 | <5.9 | nd | (43) |
| Italy | - | 118 | 83 | <101 | - | (44) |
| US coast | - | 2013 | 264 | 1409 | 321 | (45) |
| Spain | nd | **3300** | nd | nd | nd | (46) |
| Pacific Ocean | 30 | 6 | 55 | - | - | (47) |
| Germany | <20 | <7 | <46 | <18 | <25 | (48) |
| Norway | 798.7 | 439.9 | 389.9 | **7301** | - | (49) |
| Greece | nd | 19.7 | 8.2 | 10.7 | nd | (50) |

(**nd** not determined; - not mentioned)

High concentrations of UV filters were found in marine environment, showing a widespread distribution (37). In Europe, maximum concentrations up to 1043 ng/L for 4-MBC, 3316 ng/L for BP-3, 1770 ng/L for BMDBM (34), 3300 ng/L for EHMC (46), were reported in Spain and 7301 ng/L for OC in Norway (49). Similar levels were found in US

coast for BP-3, OC and BMDBM (2203 ng/L, 3730 ng/L and 1298 ng/L respectively) (32) and in Japan for BP-3 and EHMC (1340 ng/L and 1080 ng/L, respectively) (35, 40). In Hong Kong top maximum concentrations were reported by Tsui *et al*. (2014) for BP-3 (5429 ng/L), EHMC (4043 ng/L) and OC (6812 ng/L). These high levels are in the same magnitude, and even higher, than those measured in WWWTPs influents. Goksoyr *et al*. (2009) reported low levels of UV filters in open waters of the Pacific Ocean. Tsui *et al*. (2014b) reported, for the first time the occurrence of UV filters in the Artic, for which he points two possible pathways; oceanic transport via ocean currents or atmospheric transport (51). 4-MBC is not permitted for use as a cosmetic ingredient in Japan and the United States, and thus it was not detected in seawater there.

The presence of UV filters in lakes, rivers and sea associated with their lipophilic properties (Fig.4) and stability in the environment (18), indicates that they can be highly submitted to sorption in sediments. Indeed, Kameda *et al*. (2011) found maximum concentrations up to 635 ng/L (d/w) for OC in streams sediment in Japan. In sea sediment from Hong Kong, EHMC reached 447 ng/g (d/w) (51). In rivers and lakes the concentrations were at lower levels and it can be observed a widespread occurrence of EHMC and OC adsorption in sediments from different surface waters. The occurrence of these compounds in sediments could cause a potential risk to benthic organisms (52). UV filters concentrations in this kind of environmental sample depend not only on the recreational activities, but also in the number of users of sunscreens cosmetics, water tide, water renovation rate, and sampling date, among other (36).

In what concerns to the occurrence of musk fragrances in sea water few data are available. Homem *et al*. (2016) (53) recently reported maximum concentrations for galaxolide up to 336 ng/L and no detected levels of tonalide in sea water collected in Matosinhos beach, Portugal. Lower levels for galaxolide (154 ng/L) have been reported by Silva *et al*. (2010) (54) in sea water collected along Algarve seaside while tonalide reached 100 ng/L and M. ketone was below the LOD (12 ng/L).

*Biota*

UV filters have been found in tissues of natural populations of aquatic organisms such as mussels, crustaceans, eels, fishes, marine mammals and aquatic birds (Table 7). UV filters, due to their lipophilic properties, tend to accumulate in muscle and adipose tissues of aquatic organisms (3, 55). Because of the high levels found for these compounds in recreational waters and their tendency for bioaccumulation, several studies focused on the occurrence in aquatic biota (Table 7).

Bioaccumulation and biomagnification has been suggested by Fent *et al*. (2010) (55) in the predator/prey pair cormorant and fish and between the omnivorous barb feeding of Gammarus (crustacean). The aquatic bird (*Phalacrocorax* sp.) showed the highest level of EHMC (701 ng/g lw) compared with the other species sampled in the study, with EHMC concentrations ranging from 79-205 ng/g lw (Fig. 5).

High OC concentrations were reported in mussels from the Mediterranean coast (Portugal and France) reaching up to 3992 and 7064 ng/g lw (11, 56).

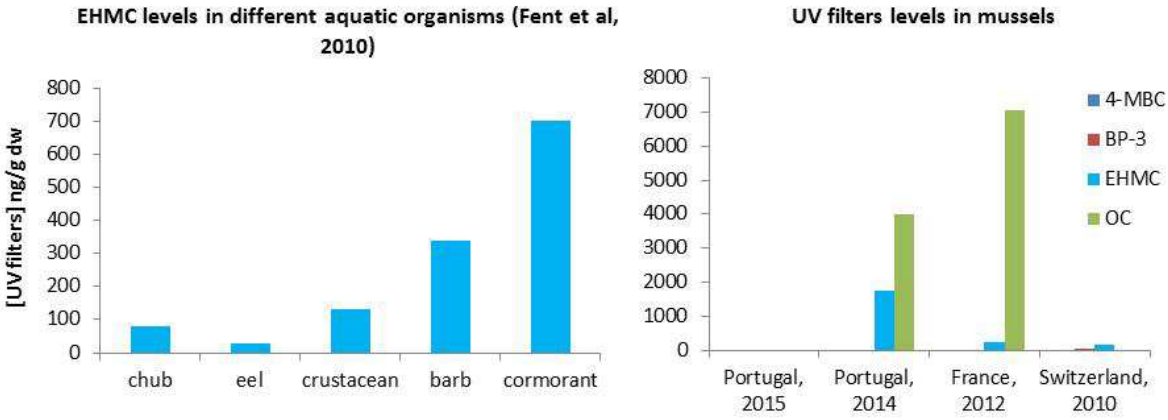



**Fig. 5** – EHMC levels in different aquatic organisms (left) and UV filters presence in mussels (right), based on data provided by Fent *et al*. (2010), Cunha *et al*. (2015), Bachelot *et al*. (2012) and Picot Groz *et al*. (2014) (11, 55-57).

However, Cunha *et al*. (2015b) (57) found BP-3, 4-MBC, BMDBM and OC in mussels from different spots in Europe, although all below the method limit of quantification (20, 5, 100 and 20 ng/g respectively) (Fig. 5). A probable cause to the low levels observed by Cunha *et al*. (2015) (58) may be the sampling campaigns dates of the referred study (September to December) that could not reflect the direct input from recreational activities. In the two other studies, the maximum concentrations were observed in July and August (11, 56). High concentrations of 4-MBC and OC were found in fresh water fish from rivers receiving inputs from WWTPs at levels up to 1800 and 2400ng/g (lw), respectively (59). Lower levels were found in lake fishes ranging from nd to 170 ng/g (lw) (4-MBC). The data indicate that river fish experience a much worse situation with respect to exposure to UV filters, than lake fish do (59). High levels of OC (782 ng/g lw) were also found in the liver of Franciscana dolphin along the coast in Brazil (60) but even higher in cod liver caught in

Norway sea (11875 ng/g lw) (61). For EHMC, the levels reported ranged 240-1765 ng/g (dw) in mussels collected in Portugal coast (11).

**Table 6** – Reported maximum concentrations of most common UV filters in tissues of different organisms (**nd** not determined; **-** not mentioned).

Fish (max. conc. ng/g dw)

| Sampling location | UV filter | | | | | | Ref. |
|---|---|---|---|---|---|---|---|
| | Common name | Scientific name | 4-MBC | BP-3 | EHMC | OC | |
| Spain (rivers) | Eel | *Anguilla anguilla* | nd | nd | <16.7 | 30 | (3) |
| | Carp | *Cyprinus carpio* | nd | 11.2 | <16.7 | nd | |
| | Barbel | *Luciobarbus sclateri* | 2.7 | 24.3 | 241.7 | 30.4 | |
| Norway (sea) | Cod | | - | 1037 | 36.9 | 11875 | (61) |
| | Prawn | | - | 68.9 | <20 | 231 | |
| | Crab | | - | <30 | <10 | <10 | |
| | Burbot | | - | <5 | <5 | <2 | |
| | Whitefish (lake) | | - | 182 | 117 | <2 | |
| | Perch (lake) | | - | 6.5 | 35.7 | 2.1 | |
| Portugal (coast) | Wild mussels | *Mytilus galloprovinciallis* | - | - | 1765 | 3992 | (11) |
| Brazil (coast) | Franciscana dolphin | *Pontoporia blainvillei* | - | - | - | 782 | (60) |
| Spain (rivers) | Barbel | *Luciobarbus sclateri* | - | 24.3 | 241.7 | 30.4 | (62) |
| | Carp | *Cyprinus carpio* | nd | 11.2 | <16.7 | nd | |
| France (coast) | Mussel | | - | - | 240 | 7064 | (56) |
| Switzerland (river) | Brown trout | *Salmo trutta* | - | 151 | 205 | - | (55) |
| | Chub | *Leuciscus cephalus* | - | <0.02 | 79 | - | |
| | Mussel | *Dreissena polymorpha* | - | <0.02 | 150 | - | |
| | Barb | *Barbus barbus* | - | <0.02 | 337 | - | |
| | Crustacean | *Gammarus* sp. | - | <0.02 | 133 | - | |
| | Eel | *Anguilla anguilla* | - | <0.02 | 30 | - | |
| | Cormorant | *Phalacrocorax* sp. | - | <0.02 | 701 | - | |
| USA (river) | Bluegill fish | *Lepomis* sp. | - | 90 | - | - | (63) |
| Switzerland (lake) | Whitefish | | <11 | <36 | 142 | - | (64) |
| Switzerland (river) | Trout | *S. trutta fario* | 1800 | - | - | 2400 | (59) |
| Switzerland (lake) | White fish | *Coregonus* sp. | 170 | - | - | nd | |
| Switzerland (lake) | White fish | | nd | nd | 72 | nd | (10) |
| | Roach | | 94 | 118 | 64 | nd | |
| | Perch | | 166 | 123 | nd | 25 | |

The use of aquatic organisms has been pointed as an integrative target sample because they are extensively exposed to hydrophobic musk fragrances and UV filters (18). Mussels are being widely used as a reliable bioindicator of chemical contamination of coastal waters because they are sessile organisms that filter and accumulate particles from water.

Musk fragrances have been detected in fish and mussels and even in mammals (Table 9). Cunha *et al*. (2015) (57) analysing mussels collected in European coast found galaxolide levels up to 34.5 ng/g (dw) and tonalide at 12.99 ng/g (dw). Celestolide was below the LOQ (5 ng/g (dw)) and no detected levels of any nitro musk. Lower levels were reported by Picot Groz *et al*. (2014) (11) and Saraiva *et al*. (2016) (65) also analyzing wild mussels collected in European coasts. High levels for galaxolide (42 ng/g) and tonalide (81 ng/g) were found by Ziarrusta *et al*. (2015) (66) for mussels collected in central America coast. In a Massachussets estuary (USA) Subedi *et al*. (2014) (19) reported levels up to 836 and 376 ng/g (dw) for galaxolide and tonalide, respectively. Similar concentrations were also detected in trouts from a German river by Lange *et al*. (2015) (26) as it can be seen in Table 9. In a monitoring study conducted by Nakata *et al*. (2012), mussels from Asia-Pacific coastal waters were collected and galaxolide and tonalide concentrations were analysed. Galaxolide levels ranged from 110 up to 3300 ng/g (dw) and tonalide from 70 to 870 ng/g (dw). The maximum concentrations were detected in mussels collected from Phillipines and Japan coast. Synthetic musks were detected in mussels from all countries, suggesting their ubiquitous contamination and widespread distribution (67).

The occurrence of the polycyclic musk fragrances galaxolide and tonalide in marine mammals and sharks collected from Japanese coastal waters was also reported (68). Galaxolide was present in the blubbers of finless porpoises (*Neophocaena phocaenoides*), at levels ranging from 13 to 149 ng/g (w/w). A fetus sample of finless porpoise contained a notable concentration of galaxolide (26 ng/g w/w), suggesting transplacental transfer of this compound. Among 12 tissues and organs of a finless porpoise, the highest galaxolide concentration was found in blubber, followed by kidney. This indicates that galaxolide accumulates in lipid-rich tissues in marine mammals.

Galaxolide was also found in the livers of five hammerhead sharks (*Sphrna lewini*) from Japanese coastal waters, at concentrations ranging from 16 to 48 ng/g w/w. Occurrence of galaxolide in higher trophic organisms strongly suggests that it is less degradable in the environment and accumulates in the top predators of marine food chains (68).

**Table 7** – Reported maximum concentrations (ng/g dw) of selected musk fragrances in tissues of different organisms.

| Sampling location | Common name | Scientific name | Musks | | | | | | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Celestolide | Galaxolide | Tonalide | M. moskene | M. xylene | M. ketone | Cashmeran | |
| European coast | mussels | | <0.001 | <1.94 | 1.23 | - | 0.013 | <0.002 | - | (69) |
| European coast | mussels | | <5 | 34.5 | 12.99 | nd | nd | nd | <2.5 | (57) |
| Central America (coast) | Wild mussels | *Mytilus edulis* | - | 42 | 81 | - | - | - | - | (66) |
| Portugal (coast) | Wild mussels | *Mytilus galloprovinciallis* | nd | 12 | - | - | - | <50 | nd | (11) |
| Tarragona (coast) | Red mullet | *Mullus surmuletus* | 6.25 | 2.97 | 1.17 | nd | nd | nd | nd | (70) |
| | Gilt head bream | *Sparus aurata* | nd | 6.12 | 3.61 | nd | nd | nd | 12.83 | |
| | Turbot | *Psetta maxima* | 8.26 | 9.67 | 5.19 | nd | nd | nd | 15.69 | |
| | Mussel | *Mytilus galloprovinciallis* | nd | 8.94 | 5.65 | nd | nd | nd | nd | |
| | Perch | *Perca fluviatilis* | nd | 18.04 | 7.53 | nd | nd | nd | 13.36 | |
| | Sheatfish | *Silurus glanis* | nd | 16.23 | 8.42 | nd | nd | nd | 33.53 | |
| | Carp | *Cyprinus carpio* | 1.56 | 12.68 | 1.38 | nd | nd | nd | 14.06 | |
| Germany (river) | Brown trout | *S. trutta fario* | - | 800 (lw) | 250 (lw) | - | | | | (26) |
| USA (estuary) Massachussets | Wild mussels | *Mytilus edulis* | - | 836 | 376 | - | - | - | - | (19) |
| Cambodia (coast) | Green and blue mussels | *Perna viridis* | - | 280 | 70 | - | - | - | - | (67) |
| China (coast) | | *Mytilus edulis* | - | 270 | 190 | - | - | - | - | |
| Hong Kong (coast) | | | - | 710 | 110 | - | - | - | - | |

Notes: nd not determined; - not mentioned); * mean of concentrations obtain in different places; ** value of 0.05ng/ml; (lw)- lipid weight basis.

**Table 7** – Reported maximum concentrations (ng/g dw) of selected musk fragrances in tissues of different organisms (cont.).

| Sampling location | Common name | Scientific name | Musks | | | | | | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Celestolide | Galaxolide | Tonalide | M. moskene | M. xylene | M. ketone | Cashmeran | |
| India (coast) | | | - | 130 | 37 | - | - | - | - | |
| Indonesia (coast) | | | - | 1500 | 180 | - | - | - | - | |
| Japan (coast) | | | - | 2300 | 860 | - | - | - | - | |
| Philippines (coast) | | | - | 3300 | 490 | - | - | - | - | (67) |
| Vietnam (coast) | | | - | 110 | nd | - | - | - | - | |
| USA (estuary) New York | Zebra mussels | | - | 19.3 (lw) | 65.9 (lw) | - | - | - | - | (71) |
| | American eel | | - | 125 | 71.5 | - | - | - | - | |
| | Channel catfish (liver) | | - | 39 | <1 | - | - | - | - | |
| | White perch | | - | 19.9 | 8.6 | - | - | - | - | |
| | Smallmouth bass (liver) | | - | 31.9 | 32.8 | - | - | - | - | |
| USA (river) Texas | Sonora sucker | | - | 569 | 58 | - | - | - | - | (63) |
| USA (river) | Common carp | | - | 1800* | 240* | - | - | - | - | (72) |
| Hong Kong (coast) | Green-lipped mussels | *Perna viridis* | - | | | - | - | - | - | |
| | mussels | | 24.6 (lw) | 1150 (lw) | 190 (lw) | - | - | - | <LOD** | (73) |

Notes: nd not determined; - not mentioned); * mean of concentrations obtain in different places; ** value of 0.05ng/ml; (lw)- lipid weight basis.

### 1.5.1. Possible adverse effects in aquatic environment

Attention has been devoted to the potential risks that PCPs may pose to aquatic ecosystems because of the worldwide contamination from widespread usage of these compounds in human activities. Information regarding UV filter toxicity is still very scarce and is not possible to develop adequate aquatic risk assessments. However, preliminary hazard assessments are already available. Some UV filters in the environment presented acute (BP-3, 4-MBC and EHMC) and chronic toxicity (BP-3, 4-MBC and EPABA) data (18). An approach to environmental risk assessment for BP-3, 4-MBC and EHMC in waters of monitoring beaches was conducted. Small potential for adverse effects for BP-3 and significant potential for adverse effects for 4-MBC and EHMC, whose risk quotient (RQ) values higher than 10, were found (34). An ecological risk assessment is available for BP-3 and although the levels observed in aquatic environment are generally an order of magnitude lower than the predicted no effect concentration (PNEC), the authors consider that further studies on environmental monitoring and potential consequences of long-term exposure in aquatic ecosystem are needed (74). 4-MBC, OC, EHMC, HMS and IMC are considered high priority for further work because of the classification with “No Observed Effect Concentration” (NOEC) values lower than 0.01 mg/L (31).
UV filters can accumulate in biota (55, 60, 62) and have been shown to cause coral bleaching (75). Some UV filters (BP-3 and EPABA) have hormonal activity in fish and display estrogenic and activity in vitro (76). It was also found that mixtures of UV filters showed synergistic interactions in vitro and additive to antagonistic activity in vivo (55).

The toxicity of galaxolide and tonalide to aquatic biota has been studied in several organisms. Inhibition of larval development in the crustacean *Acartia tonsa* was reported with $EC_{50}$ values of 0.026 mg/L (galaxolide) and 0.059 mg/L (tonalide) (77). Estrogenic effects of polycyclic musks have been shown in in vitro competitive binding assays with South African clawed frogs (*Xenopus laevis*) and rainbow trout (*Onchorynchus mykiss*) (78). Anti-estrogenic effects of galaxolide and tonalide have also been reported in zebrafish (*Danio rerio*) in both in vivo and in vitro studies (79) as well as inhibition of multi-xenobiotic defences of marine mussel *Mytilus californianus* gill cells (80). Balk and Ford (1999) (81) determined PNECs of 6.8 g/L and 3.5 g/L for galaxolide and tonalide, respectively, in early life stage tests with fathead minnow *Pimephales promelas*. The results of these studies indicate that polycyclic musks pose potential risks to aquatic organisms, but there is still a lack of toxicological information available for assessing the risks of the compounds to aquatic ecosystems.

Galaxolide and tonalide were the most frequently detected polycyclic musk compounds in a number of fish, mussel, shrimp and crab samples from the marine environment (Table 7).

### 1.5.2. Possible adverse effects in humans

The increased usage of UV filters-containing PCPs must be studied critically, not only because of the increasing release of the UV filters into the environment and their possible ecological impacts, but also due to their behaviour on the skin (5).

The reason why these compounds are under the scope is related to their toxicity and adverse effects, being the most problematic: (i) permeation into the viable layers of the skin; (ii) interference with the endocrine system in humans; (iii) photounstability (82).

Given that sunscreen formulations are applied to a large skin area for a long period of time, a constant and high input of organic UV filters into skin viable cell layers and into systemic circulation are permitted (83). Calculations on amount of UV filter compounds per application suggested by Balmer *et al*. (2005) (10), assuming a use of 10 g of sun cream for a full body application (1 mg per $cm^2$ skin surface), and 2-20 % content of UV filters in a sunscreen product, this amounts to 0.5 to 2 g per day (10).

A large number of in vivo animal studies and in vitro studies have shown potential adverse effects like endocrine disrupting of UV filters present in sunscreens, although other studies failed to find such effects. Application of cosmetics with UV filters to the skin can result in absorption of UV filters in the human systemic circulation directly without first being metabolized by passage through the liver, leading to a greater risk of the compounds reaching all tissues of the body unaltered resulting in exposure of all tissues in the body (84).

Based on the median concentrations of musks and the average daily usage amounts of consumer products, dermal exposure rates in adults were calculated to be 3.38 mg/d for musks (85). Galaxolide was found in human adipose fat collected from New York City, at concentrations ranging from 6.1 to 435 (mean: 97) ng/g, on a wet weight basis. Tonalide was found in 86% of the samples analyzed, at concentrations ranging from <5 to 64 (mean: 23) ng/g (ww) (86). Occurrence of galaxolide and tonalide in human adipose tissues from Germany and Switzerland was also reported (87) as well as in human milk samples from Germany, Denmark and USA (87-90). Dermal absorption from the use of PCPs is thought to be a major source of human exposure to these compounds (86).

### 1.6. Assessment of Musk fragrances and UV filters contamination in aquatic biota

Monitoring of PCPs in aquatic environments (waters, biota and sediments) has been reported in several countries, such as Switzerland, China, Brazil, Japan, Italy, among others, as it was shown in section 1.5. Sensitive and selective analytical methods, capable of detecting PCPs at trace levels in aquatic biota, have been reviewed by Gago-Ferrero *et al*. (2012) (22). Methodologies for the determination of UV filters in environmental samples have been previously reviewed by Giokas *et al*. (2007) and Peck *et al*. (2006) (1, 91).
In this scenario, where the number of pollutants to be monitored is getting larger, multi-residual analytical methodologies become an excellent strategy to get this goal.

*Precautions to avoid sample contamination*

Considering the widespread use of PCPs at high concentrations in virtually everything used in daily life (perfumes, soaps, creams, cosmetics, shampoo, lipstick and sunscreens, etc.) laboratory contamination appeared to be imminent. Background contamination is a common problem in the determination of UV filters and musks fragrances at environmental levels (92). Due to their lipophilic nature, these compounds are easily transferred to glassware and consumables used during sampling and sample preparation. Precautions must be taken to prevent contamination from personnel, equipment and glassware (93). The use of plastic material should be avoided (94). Relating to personnel contamination, avoiding the use of any PCPs during either sampling or analytical procedures (91, 95); use of gloves during all procedures, pre-cleaning of sampling containers, sampling and sample processing (92, 93, 95). Careful cleaning of glassware is important, providing glassware exclusively for the analysis of UV filters (93), sonication with a detergent without these chemicals, soaking in 5 % NaOH/ ethanol (EtOH) for 12 h (96) or a 50 % $HNO_3$ solution (33), baking for at least 4 h at 450 ℃ ( 91), 3h in muffle type furnace at 500 ℃ (58, 64), heated overnight at 380 ˚C (92), and further sequentially rinsed with a collection of organic solvents (acetone (ACN), n-hexane, methanol (MeOH), dichloromethane (DCM), acetonitrile (MeCN)) and HPLC grade water. High-purity solvents and only previously unopened packages should be used (91, 92). Prior to use, all glassware should be rinsed with the extraction solvent (97). Attention should be also paid to equipments involved in extraction procedures, for example with stir bars used in Stir Bar Sorptive Extraction (SBSE) re-conditioning before re-using at 250 ℃ for 3 h (98) or soaking in a mixture of DCM/MeOH (1:1, v/v) for 24 h (44). Procedural blanks should be used to monitor for contamination from the laboratory environment, instrumentation, contaminated solvents (91), sampling or storage (58). Since many of the compounds

analyzed undergo photodegradation, stock standard solutions and samples should be covered with aluminium foil and stored in the dark or in amber glass (92).

### 1.6.1. Sampling and pre treatment

Aquatic organisms can be quite representative of the aquatic environment as they can retain and bioaccumulate PCPs because of the lipophilicity of the compounds (log $K_{ow}$ close to 6) (55, 62). Most studies have focused on fish as a representative matrix. Sampling procedures may involve traditional fishing, trawling (cod and shrimp) snorkelling (crab) (61) electrofishing (3), Direct Current (DC) electric pulse (62) and even incidental caught in fishing nets or special situations like individual dolphins found stranded dead at the beaches (60). After being caught, they are killed, weighed, measured, wrapped in aluminium foil, kept frozen until laboratory. Selected tissues are removed, frozen, homogenized by blending and often freeze-dried before extraction (22). Muscle is often used for analyses of target compounds, probably because of its low lipid content in comparison with other tissues and because it is part of the human diet. Studies have also been conducted on macrozoobenthos, mussels, and birds (55).

Mussels are good biomonitor organisms for detection of water pollutants as they constantly filter the surrounding waters. Difficulties related to intra species diversity in natural waters, length, weight, lipid content, sex, and availability of similar species in different habitats hinder the comparison of results. Nevertheless, their wide distribution, easy to sample, high abundance, low mobility, and ecological and economic importance are features that make them a good choice to monitor the contaminants.

### 1.6.2. Extraction and clean up procedures

In the biomonitoring of coastal environments many extraction techniques have been applied to mollusc samples. Due to the very low concentrations of PCPs in the environment, extraction usually requires a preconcentration and clean up step previous to the analysis in order to achieve low limits of detection (LODs) and eliminate some potentially interfering compounds.

Extraction and purification are key steps in decreasing matrix effects and optimizing analytical efficiency (11). Pressurized liquid extraction (PLE), solid–liquid extraction (SLE), and microwave-assisted extraction (MAE) were reported as the major approaches to extract target compounds from their matrices (22). DCM, ACN and mixtures of ethyl acetate (EtAC)/heptane and $H_2O$ (1:1:1) or ACN/heptane (1:1) have been used as solvents in PLE, LE and MAE extractions of musks and UV filters from aquatic biota (55, 56, 61, 62). PLE remains at the top, followed by liquid-liquid extraction (LLE), Quick, easy,

cheap, effective, rugged and safe (QuEChERS) and MAE (Fig. 6). It is worth noting that these techniques lead to coextraction of a lipid fraction that should be removed before determination of musks and UV filters. Clean-up of biota sample extracts can be done by gel permeation chromatography (GPC) (61, 63) primarily to remove lipids, followed by addition of primary-secondary amine (PSA) sorbent that has strong affinity and high capacity for removing fatty acids and organic acids (11, 61), adsorption chromatography on silica (63) or Florisil columns (62). Quite often reverse phase – high performance liquid chromatography (RP-HPLC) has also been used for extraction and purification (55, 56, 64). Although some of these techniques are now automatized, they are long, time consuming and require a large volume of solvents. QuEChERS procedures have been modified to the determination of PCPs in mussels, followed by dispersive liquid-liquid extraction (DLLME) (57) or a clean-up sorbent (a mixture of 750 mg $Na_2SO_4$, 125 mg Bondesil-$C_{18}$ and 125 mg PSA silica) (11). Lipid content determination is important and it´s usually performed gravimetrically (55, 56).

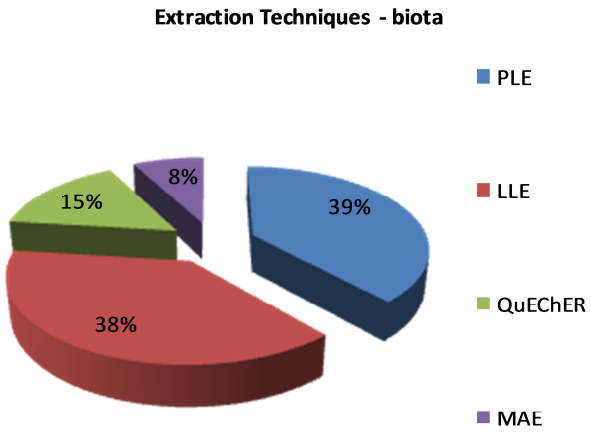



**Fig. 6** – Extraction techniques employed in aquatic biota samples for the determination of musk fragrances and UV filters.

### 1.6.3. Instrumental analysis

After performing extraction and clean up procedures to eliminate interfering compounds from the matrix, an appropriate analytical method must be selected in order to enhance identification and quantification of the target compounds in environmental samples (21). Usually, chromatographic methods, both gas (GC) and liquid chromatography (LC), coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS) detectors, allowed the sensitivity required to detect trace levels of the potential contaminants. The

selection of GC or LC is usually based on the physical and chemical properties of the analytes and the availability of the equipments in the laboratories facilities. LC is selected to determine more polar and less volatile compounds, while GC is used to quantify volatile or volatile compounds (99).

#### 1.6.3.1. Gas Chromatography

Most of the analytical methods described in the literature for musks and UV filters analysis in aquatic biota are based on GC-MS (Tables 8 and 9), after an extraction and clean up procedure, usually by PLE or LLE. QuEChERS approach is arising in this kind of environmental analysis (Fig. 6). Since the UV filters contain phenolic hydroxyl groups, reproducibility and sensitivity with GC analysis can be affected due to their insufficient volatility, high polarity and thermal instability, so a derivatization step prior the GC injection can be applied to obtain sharper peaks, better separation and higher sensitivity (94). Derivatization improves volatility, thermal stability and other desirable chromatographic features of the target analytes (58). Higher sensitivity is achieved, preventing co-elution with matrix interferences, by increasing the molecular weight of the molecule, with a subsequent increase in the retention time and reduction in polarity of the analyte (96). Derivatization can be used to expand the applicability of GC/MS and GC–MS/MS analyses to more polar compounds (4). Silylation is by far the most used derivatization method, among the different derivatization strategies (e.g., silylation, alkylation, esterification, acylation, etc.), for compounds containing labile hydrogens, since the derivatization process can be easily achieved and there are a large number of silylation reagents available. When a silylation reagent is used, the labile hydrogens of the compound are replaced by alkylsilyl moieties, usually trimethylsilyl. Thus, the OH moieties are turned into their trimethylsilyl ethers, which are more volatile than their parent compounds (46). N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) (38, 100, 101), and N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) (43, 57, 58) (46, 96) are the most common derivatizing agents used for silylation of UV filters. When comparing the efficiency of both derivatizing agents, BSTFA showed better results, for example with higher peak area (46) and high intensity chromatographic response for compounds containing a labile H (OH) such as hidroxylated UV filters, probably due to its high reactivity (58) analyzing water samples. Even though, MSTFA was employed by several authors to determine UV filters biota samples (Table 9). Usually derivatization procedure is conducted under high temperatures (60 ℃) and up to 1 h of reaction time (38, 100), being a time-consuming step, considered a drawback for GC UV filters analysis. Microwave (MA) or ultrasonic assisted (UA) derivatization procedures coupled to DLLME

extraction technique, can overcome this inconvenience reducing the time required for derivatization to 5 or even 2 min (58, 96), performing an *in situ* derivatization resulting in effectively shorten the overall analysis time (58). Negreira *et al*. (2009) (101), performed on-fiber silylation with MSTFA, using SPME for 30 min. Another drawback of derivatization is that it requires the use of reagents that may be highly toxic and carcinogenic.

Even though, it´s important to note that some compounds, namely 4-MBC, EHMC, OC and BMDBM can be detected without derivatization. Cunha *et al*. (2015) (58) compared the analytical response obtained from the direct injection with those after the derivatization step, and found no differences between both, proving that, for the referred UV filters, there were no losses during the derivatization procedure. Musks fragrances usually don´t require derivatization. Multi-residue methods are ideally preferred taking into account the huge amount of compounds of interest, so derivatization of the sample can enable a single injection per sample covering a wide range of compounds (58).

Furthermore, GC-MS with selective ion monitoring (SIM) or GC-MS/MS have been the most frequent working mode to efficiently remove interference from co-eluting peaks and to ensure sensitivity, facilitating the detection of trace levels of organic compounds (11).

**Table 8** – Extraction techniques coupled to the GC analysis of UV filters in aquatic biota samples, recoveries, limits of detection (LOD) and limits of quantification (LOQ) (- not mentioned).

| Species | Extraction technique | Derivatization | Analytical technique | Column (length x id.; film thickness) | UV filters | Recovery (%) | LOD (ng/g lw) | LOQ (ng/g lw) | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| Mussels | **QuEChER-DLLME** | **50 µl BSTFA** | **GC-MS** | DB-5MS | BP-3 | 72-83 | 3 | 20 | (57) |
| | | | | 30 m x 0.25 mm; 0.25 | | | | | |
| | | 5 min, MW (600W) | | µm | 4-MBC | 79-96 | 2 | 5 | |
| | | | | | OC | 75-76 | 23 | 100 | |
| | | | | | BMDBM | 60-61 | 6 | 20 | |
| Fish | **PLE-GPC-PSA** | | **GC/HRMS** | DB-5MS | BP-3 | - | 5-30 | - | (61) |
| | | | | 30 m x 0.25 mm; 0.25 | | | | | |
| | | | EI + | µm | EHMC | - | 5-20 | - | |
| | | | | | OC | - | 2-10 | - | |
| Wild mussels | **QuEChER** | | **GC/MS** | fused silicaZB-5 | EHMC | 93-106 | 1 | 5 | (11) |
| | | | | 30 m x 0.25 mm; 0.25 | | | | | |
| | | | | µm | OC | 99-126 | 5 | 5 | |
| Wild mussels | **MAE-RP-HPLC** | | **GC-MS/MS** | SGE-BPx5 | EHMC | 88-99 | 2 | 5 | (56) |
| | | | | 30 m x 0.25 mm; 0.25 | | | | | |
| | | | | µm | OC | 89-101 | 2 | 5 | |
| Fish | **LE-RP-HPLC** | | **GC/MS** | optima-5-MS | BP-3 | 70-105 | | - | (55) |
| | | | | 50 m x 0.2 mm; 0.35 | | 70-106 | 0.005-0.02 | - | |
| Macroinvertebrate | | | | µm | EHMC | | | | |
| Cormorant | | | | | 4-MBC | 70-107 | | - | |
| Fish | | | **GC/MS** | Optima-5-MS | BP-3 | 76.1-98.9 | 36 | - | (64) |
| | | | | 50 m x 0.2 mm; 0.35 | | 76.1-98.10 | | - | |
| | | | | µm | EHMC | | 11 | | |
| | | | | | 4-MBC | 76.1-98.11 | 23 | - | |
| Fish | **LLE-silica** | **100 µl MSTFA** | **GC/MS** | XTI-5 | 4-MBC | 99 | 5.3 | - | (63) |
| | | | | 30 m x 0.25 mm; 0.25 | | | | - | |
| | | **45 min, 60 °C** | EI | µm | OC | 98 | 17 | | |
| | **LLE-GPC** | | | VF-5 | 4-MBC | 57 | 120 | - | |
| | | | | 30 m x 0.25 mm; 0.25 | | | | - | |
| | | | | µm | OC | 79 | 36 | | |

**Table 9** – Extraction techniques coupled to the GC analysis of musks in aquatic biota samples, recoveries, limits of detection (LOD) and limits of quantification (LOQ).

| Species | Extraction technique | Analytical technique | Column (length x id.; film thickness) | Musks | Recovery (%) | LOD (ng/g lw) | LOQ (ng/g lw) | Ref. |
|---|---|---|---|---|---|---|---|---|
| Wild mussels | matrix solid-phase dispersion (MSPD) | **GC-MS** | HP-5MS 30 m x 0.25 mm; 0.25 µm | Galaxolide | 116 | 14 | - | (66) |
| | | | | Tonalide | 45 | 29 | - | |
| | | **GC-MS/MS** | | Galaxolide | 123 | 6.3 | - | |
| | | | | Tonalide | 45 | 4.1 | - | |
| Wild mussels | **QuEChERS** | **GC-MS/MS** | fused silica ZB-5 30 m x 0.25 mm; 0.25 µm | Galaxolide | 89 | 0.5 | 0.5 | (11) |
| | | | | Cashmeran | 84 | 50 | 50 | |
| | | | | M. Ketone | 97 | 50 | 50 | |
| | | | | Celestolide | 81 | 0.5 | 2.5 | |
| Wild mussels | **QuEChERS** | **GC-IT-MS/MS** | ZB-50 30 m x 0.25 mm; 0.25 µm | Celestolide | 64 | 2.5 | 5 | (70) |
| | | | | Galaxolide | 62 | 0.5 | 2.5 | |
| | | | | Tonalide | 59 | 0.5 | 2.5 | |
| | | | | M. Moskene | 25 | 7.5 | 20 | |
| | | | | M. Xylene | 57 | 7.5 | 20 | |
| | | | | M. Ketone | 24 | 7.5 | 20 | |
| | | | | Cashmeran | 110 | 1 | 5 | |
| | | | | Celestolide | 88 | 1 | 5 | |
| | **PLE** | | | Galaxolide | 91 | 2.5 | 2.5 | |
| | | | | Tonalide | 86 | 1 | 2.5 | |
| | | | | M. Moskene | 57 | 5 | 10 | |
| | | | | M. Xylene | 67 | 5 | 10 | |
| | | | | M. Ketone | 54 | 5 | 10 | |
| | | | | Cashmeran | 79 | 0.5 | 2.5 | |

Notes: (*) Limit of detection expressed in ng/ml; (nd) not determined; (-) not mentioned); (lw)- lipid weight basis.

**Table 9** – Extraction techniques coupled to the GC analysis of musks in aquatic biota samples, recoveries, limits of detection (LOD) and limits of quantification (LOQ) (cont).

| Species | Extraction technique | Analytical technique | Column (length x id.; film thickness) | Musks | Recovery (%) | LOD (ng/g lw) | LOQ (ng/g lw) | Ref. |
|---|---|---|---|---|---|---|---|---|
| Brown trout | **Soxhlet extraction** | **GC-MS** | VF-XMS | Galaxolide | - | - | 1 | (26) |
| | | | 30 m x 0.25 mm; 0.25 µm | Tonalide | - | - | 1 | |
| mussels | **LE-Soxhlet** | **GC/MS** | Rxi-5-MS | Galaxolide | 76-101 | - | 0.1-10 (lw) | (19) |
| | | | 30 m x 0.25 mm; 25 µm | Tonalide | 76-101 | - | 0.1-10 (lw) | |
| mussels | **LE-GPC** | **GC/MS** | HP 5-MS fused silica | Galaxolide | 114 | 0.8 (ww) | - | (67) |
| | | | 30 m x 0.25 mm; 0.25 µm | Tonalide | 92 | 0.4 (ww) | - | |
| mussels | **LE-GPC** | **GC/MS** | DB 5-MS fused silica | Galaxolide | 85-98 | - | 1 | (71) |
| fish | | | 30 m x 0.25 mm; 0.25 µm | Tonalide | 85-98 | - | 1 | |
| Blue gill fish | **LLE-silica** | **GC/MS** | XTI-5 | Celestolide | 83 | 18 | - | (63) |
| | | | | Galaxolide | 95 | 12 | - | |
| Sonora sucker | | EI | 30 m x 0.25 mm; 0.25 µm | Tonalide | 107 | 13 | - | (72) |
| | **LLE-GPC** | **GC-MS/MS** | VF-5 | M. Xylene | 67 | 397 | - | |
| | | | 30 m x 0.25 mm; 0.25 µm | M. Ketone | 75 | 321 | - | |
| Green-liped mussels | **LE-GPC** | GC/MS | DB 5HT fused silica | Celestolide | 76.2 | 0.05* | - | (73) |
| | | | 30 m x 0.25 mm; 0.25 µm | Galaxolide | 94.1 | 0.09* | - | |
| | | | | Tonalide | 79.5 | 0.14* | | |
| | | | | Cashmeran | 72.5 | 0.15* | | |

Notes: (*) Limit of detection expressed in ng/ml; (nd) not determined; (-) not mentioned); (lw)- lipid weight basis; (ww)- wet weight basis.

### 1.6.3.2. Liquid Chromatography

LC-MS/MS may be considered the technique of choice to assay polar and semipolar compounds, and is especially suitable for environmental analysis because of its selectivity (102). It allows separation and detection of compounds having the same molecular mass but different product ions, even if they co-elute. MS/MS detection is therefore preferred for increased analytical sensitivity and selectivity in complex water matrices (4). In addition to HPLC, ultra performance liquid chromatography (UPLC) is being used increasingly (34, 103-106). UPLC uses analytical columns packed with smaller particles, which offers the advantages of increasing speed, improving sensitivity and mostly selectivity compared to conventional HPLC analysis. The higher efficiency of small particles enables shorter columns, reducing analysis time and solvent consumption. Although complete chromatographic separation is not necessary for the selective MS/MS detection, it generally improves detectability and reduces the ion suppression effect (107).

Reverse phase $C_{18}$ HPLC columns are the most used in the analysis of UV filters by LC-MS/MS (Table 10). Most common mobile phase solvents are MeOH and water (42, 108-111). However, the use of MeCN and water is also very common and has been employed in several works (64, 112, 113). In order to increase ionization and sensitivity ammonium acetate (48, 111, 113, 114) and formic acid (42, 64, 112-114) is often included as a mobile phase additive, at concentrations typically between 0.05% and 0.3% (102).

Atmospheric pressure ionization (API) technologies, such as atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization sources (APPI) and electrospray ionization (ESI) are commonly employed as ionization techniques in LC-MS/MS. APCI is very effective in the analysis of medium-polarity and low-polarity substances, whereas ESI (positive or negative ionization mode) achieves efficient ionization for a great variety of analytes. Nonetheless, API interfaces can lead to problems due to its susceptibility to matrix effects (92). Matrix effects can strongly vary with the matrix and result in poor analytical accuracy and reproducibility with co-extracting of matrix components. The use of isotopically labelled surrogate standards (115) or internal standard quantification with matrix-matched standards (112) is employed to compensate this negative effect. APCI was shown to be less susceptible to ion suppression, but with decreased sensitivity (113).

For the analysis of UV filters, LC-MS/MS offers an improvement over GC-MS since the derivatization step is avoided for polar and semi-polar compounds and LODs in the low ng/L or ng/g can still be achieved (Table 10).

Researchers have used different analytical approaches for the environmental analysis of musks and UV filters, based on SPE for aqueous sample extraction and PLE or SLE for solid samples and with further clean-up protocols. In the case of PLE the clean-up can be performed together with the extraction, reducing laboriousness and time consumption. In order to reduce solvent consumption, new approaches have been developed such as QuEChERS and DLLME. Both HPLC and UPLC attached to mass spectrometry are employed using preferably ESI but also APCI or APPI.

**Table 10** – Extraction techniques coupled to the LC analysis of UV filters in aquatic biota samples, recoveries and limits of detection (LOD) and limits of quantification (LOQ).

| Species | Extraction technique | Analytical technique | Column (length x id.; film thickness) | UV filters | Recovery (%) | LOD (ng/g lw) | LOQ (ng/g lw) | Ref. |
|---|---|---|---|---|---|---|---|---|
| Fish | **PLE-SPE** | **LC-MS/MS** | HR R-18 | BP-3 | 106-112 | 1.2 | 4 | (3) |
| | | ESI + | 50 mm x 2 mm; 5 µm | EHMC | 66-72 | 5 | 16.7 | |
| | | | ESI + | 4-MBC | 95-109 | 0.7 | 2.3 | |
| | | | | OC | 70-80 | 6 | 20 | |
| Fish | **PLE-GPC** | **LC-HRMS** | BEH - C8 | BP-3 | 75 | - | 20 | (61) |
| | | | 50 mm x 2.1 mm | EHMC | 85 | - | 30 | |
| | | | | OC | 75 | - | 20 | |
| Dolphin | **PLE-SPE** | **LC-MS/MS** | HR R-18 | OC | - | 23 | 75 | (60) |
| | | | 50 mm x 2 mm; 5 µm | | | | | |
| Fish | **in cell PLE-SPE** | **LC-MS/MS** | HR R-18 | BP-3 | 106-112 | 1.2 | 4 | (62) |
| | | ESI | 50 mm x 2 mm; 5 µm | 4-MBC | 95-109 | 0.7 | 2.3 | |
| | | | | EHMC | 66-72 | 5 | 16.7 | |
| | | | | OC | 70-80 | 6 | 20 | |
| Fish | **lipophilic analytes: LLE-RP-HPLC hidrophilic analytes: LLE** | **LC-MS** | Zorbax SB - C18 150 mm x 3 mm; 3.5 µm | BP-3 | 70-105 | 0.2-0.4 | - | (55) |
| Macroinvertebrate | | | | EHMC | | | - | |
| Cormorant | | | | 4-MBC | | | - | |
| | | **LC-MS** | Zorbax SB - C18 150 mm x 3 mm; 3.5 µm | BP-3 | 76.1-98.9 | - | - | (64) |
| | | | | EHMC | | - | - | |
| | | | | 4-MBC | | - | - | |

(- not mentioned)

## 1.7. Aim of the study

Musk fragrances and UV filters have come into focus in the last years due potentially harmful concentration in aqueous environment worldwide. The main objective of the present work was to adapt and validate a method based on QuEChERS followed by DLLME extraction and GC–MS analysis for the simultaneous determination of musks and UV filters in wild mussel samples collected along the coastal line of Portugal, in order to evaluate the real contamination of these aquatic organisms continually exposed in their habitat to this kind of contaminants.

# Chapter 2

## MATERIALS AND METHODS

## 2.1. Standards and Reagents

2-Hydroxy-4-methoxybenzophenone (BP3, 98% purity) and 2-ethylhexyl-4-(dimethylamino) benzoate (EPABA, 98% purity) were purchased from Alfa Aesar (Heysham, Lancashire, UK). 3,3,5-trimethylcyclo-hexylsalicylate (HMS, 98 % purity) and isoamyl-4 methoxycinnamate (IMC, 95% purity) were purchased fromTCI (Haven, Zwijndrecht, Belgium). Octocrylene (OC, 98% purity), 2-ethylhexyl 4-methoxycinnamate (EHMC, 100% purity), 2-ethylhexylsalicylate (EHS, 99 % purity), hexyl 2-[4-(diethylamino)-2-hydroxybenzoyl] benzoate (DBENZO, 99% purity), 2,4-dihydroxybenzophenone (DHB, 99% purity) 3-(4-methylbenzylidene) camphor (4-MBC, 98.5% purity), Butyl-methoxydibenzoylmethane (BMDBM, 100% purity) 6,7-dihydro-1,1,2,3,3-pentamethyl-4(5H)-indanone (DPMI, cashmeran), 4-acetyl-1,1-dimethyl-6-tert-butylindane (ADBI, celestolide, 98.5% purity), 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(g)-2-benzopyran (HHCB, galaxolide, 97% purity) and 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetra- hydronaphthalene (AHTN, tonalide 98.5% purity), 2,4,6-trinitro-1,3- dimethyl-5-tert-butylbenzene (MX, musk xylene), 4-aceto-3, 5-dimethyl-2,6-dinitro-tertbutylbenzene (MK, musk ketone) and 1,1,3,3,5- pentamethyl-4,6-dinitroindane (MM, musk moskene) were purchased from Sigma-Aldrich (Steinheim, Germany).

The internal standard (IS) Chrysene d-12 (CSd12-IS1, 98% purity) and Benzophenone-d10 (BPd10-IS2, 99% purity) and were also purchased from Sigma-Aldrich.

Individual standard solutions of the UV filters were prepared in methanol (HPLC grade from Sigma-Aldrich) at concentrations of 2000 mg/L. Working mixture solutions of 100 mg/L were prepared in acetonitrile, the solvent used in the extraction. Individual standard solutions of the synthetic musk fragrances were prepared in acetone at concentrations of 4000 mg/L for polycyclic musks and 1000 mg/L for musk ketone. A working mixture solution of 100 mg/L was prepared in acetonitrile except for MX, MM which were supplied directly at a concentration of 100 mg/L in acetonitrile and used as received.

Acetonitrile (MeCN), methanol (MeOH), acetone (ACN), all HPLC grade, were obtained from Sigma–Aldrich. Extractive solvents, trichloroethylene ($C_2HCl_3$) and carbon dissulfite ($CS_2$), were high purity solvents for GC analysis obtained from Fluka (Neu-Ulm, Germany). The sorbents sulphate magnesium ($MgSO_4$) and sodium chloride were both obtained from Sigma-Aldrich. To ensure efficient removal of phthalates and residual water, $MgSO_4$ was treated for 5 h at 500 ℃ in a muffle furnace.

Hydrochloric acid and pH test strips (0–14 pH resolution: 1.0 pH unit) were purchased from Sigma-Aldrich. Water was prepared by purifying demineralized water in a “Seradest LFM20” system (Seral, Ransbach-Baumbach, Germany). Ultrahigh purity Helium (99.999%) for GC–MS was purchased from Gasin (Maia, Portugal).

### 2.2. Sampling

Mussels (*Mytilus galloprovincialis* and *Mytilus edulis*) were collected by hand between January and October 2015 in seven sites of the Portuguese coastal. When in laboratory, total weight (g) and edible weight (g) of mussel specimens were registered (Table 11). Then, all the edible content of 25 specimens were pooled, triturated/homogenized by a grinder (Retasch Grindomix GM200, Germany), and frozen at -80 ℃ in plastic tubes of 40mL. To conclude, the samples were freeze-dried for 48 h at -80 ℃ and low pressure (around 0.017 mBar, Telstar Cryodos, Grundy's LaneBristol), homogenized as above, and maintained at 4 ℃ until analysis.

**Table 11** - Description of the mussel specimens collected along Portuguese beaches.

| Sites | Edible weight (total weight) g | | | | |
|---|---|---|---|---|---|
| | January | March | May | July | October |
| **Viana do Castelo** | 1.24-2.95 (-) | 0.94-4.39 (-) | 0.89-2.32 (3.33-7.03) | 1.13-2.08 (3.69-8.57) | 1.95-3.54 (6.30-13.56) |
| **Leça de Palmeira** | 3.48-6.49 (-) | 1.59-6.79 (-) | 1.17-3.09 (4.20-13.45) | 1.05-3.51 (3.97-10.74) | 1.34-3.35 (4.44-8.72) |
| **Vagueira** | 0.73-2.29 (-) | 0.77-2.03 (-) | 2.31-4.92 (1.01-2.59 ) | 1.39-2.77 (3.81-9.90) | 1.30-5.50 (5.397-12.34) |
| **Algés** | 1.42-8.49 (-) | 0.95-5.77 (-) | 0.73-2.03 (3.41-6.11) | 2.34-4.61 (7.96-20.99) | 1.85-5.96 (7.05-24.42) |
| **Costa da Caparica** | 1.42-2.97 (-) | 4.96-12.99 (-) | 1.91-5.53 (5.10-11.27) | 2.25-8.0 (10.53-33.94) | 2.02-5.26 (7.23-11.48 ) |
| **Aljezur** | 3.80-18.76 (-) | 3.67-8.37 (-) | 0.96-5.78 (6.31-25.65) | 1.14-9.50 (2.81-26.61) | 2.53-6.90 (7.39-14.29) |
| **Faro** | 1.33-18.92 (-) | 5.63-12.66 (-) | 5.15-19.21 (2.06-5.53) | 7.02-19.86 (2.27-5.17) | 1.89-4.41 (14.14-23.14) |

(-) not mesuared


**Fig. 7 -** Location of the sampling spots along the Portuguese coast.

### 2.3. GC-MS conditions

The gas chromatograph 6890 (Agilent, Little Falls, DE, USA) equipped with a Combi-PAL autosampler (CTC Analytics, Zwingen, Switzerland) and an electronically controlled split/splitless injection port was interfaced to a single quadrupole inert mass selective detector (5975B, Agilent) with electron ionization (EI) chamber. GC separation was performed on a DB-5MS column (30 mx 0.25 mm i.d. 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA). Helium was the carrier gas with a constant flow of 1 mL/min. The injection was made in splitless mode (purge-off time 60 s) at 250℃. The oven temperature programme was as follows: 90℃ held for 1 min, ramped to 150℃ at 20℃/min, ramped to 225℃ at 5℃/min and then rampe d 300℃ at 20℃/min held for 5.25 min. Total run time was 28 min. The MS transfer line was held at 280℃. Mass spectrometric parameters were set as follows: electron ionization with 70 eV energy; ion source temperature, 230℃ and MS quadrupole tempera ture, 150℃. The MS system was routinely set in selective ion monitoring (SIM) mode and each analyte was quantified

based on peak area using one target and two qualifier ion(s). Complete SIM parameters and retention times of the analytes are shown in Table 12. Agilent Chemstation was used for data collection/processing and GC–MS control.

**Table 12 –** Retention times and MS conditions for the GC–MS analysis of musks and UV filters.

| Family/compound | | $t_R$ (min) | time windows | SIM ions m/z[a] |
|---|---|---|---|---|
| *Musks* | **Celestolide** | 10.88 | 10.6 | **229**, 244, 173, 230 |
| | **Galaxolide** | 13.19 | 13.0 | **243**, 258, 282, 213 |
| | **Tonalide** | 13.41 | 13.0 | **243**, 258, 159, 187 |
| | **M. moskene** | 13.90 | 13.7 | **263**, 278, 128, 264 |
| | **M. xylene** | 13.45 | 13.0 | **282**, 297, 243, 128 |
| | **M. ketone** | 15.61 | 15.1 | **279**, 294, 280, 128 |
| | **Ethylene brassylate** | 16.11 | 15.9 | **227**, 155, 211, 187 |
| *UV filters* | **EHS** | 12.36 | 12.0 | **120**, 138, 250, 92 |
| | **BP-3** | 16.61 | 16.4 | **227**, 151, 228, 77 |
| | **4-MBC** | 16.92 | 16.4 | **254**, 211, 171, 239 |
| | **IMC** | 16.57 | 16.4 | **178**, 161, 248, 133 |
| | **BMDM** | 23.91 | 23.5 | **310**, 135, 161, 295 |
| | **DBENZO** | 26.30 | 26.0 | **382**, 397, 268, 383 |
| | **HS** | 13.86 | 13.7 | **138**, 120, 121, 262 |
| | **EPABA** | 20.18 | 19.7 | **165**, 148, 277, 164 |
| | **EHMC** | 20.68 | 20.4 | **178**, 161, 290, 133 |

[a]Quantification ions are shown in bold type

## 2.4. Statistical Analysis

The relationships between concentrations of musks and UV filters, and between each other, in mussels, were examined using Spearman's rank correlation analysis. A *p* value <0.05 was considered statistically significant. In order to monitor the fluctuations in the occurrence of the target compounds in the studied area, a spatial distribution and seasonal variation was evaluated. All statistical analyses were performed with the aid of IBM SPSS Statistics version 24 for Windows.

# Chapter 3

## RESULTS AND DISCUSSION

### 3.1. Sample optimization

Based in previous works developed by the research group in which I was integrated, a procedure including QuEChERS extraction followed by DLLME was chosen for the simultaneous extraction of musk fragrances and UV filters from wild mussels.

QuEChERS is considered a “green” analytical approach because it combines low utilization of solvents with a quick procedure with low waste production. DLLME is another recently developed extraction technique presenting unique features in what concerning simplicity of operation, amount of organic solvent extractor (only a few microliters), quickness, and high enrichment factor. Overall, DLLME is a very suitable technique for the extraction/enrichment of compounds with some hydrophobicity prior to their determination by GC.

The combination of QuEChERS with DLLME enables a rapid and inexpensive sample treatment that ensures a high enrichment factor and consequently good detection limits (116). However, in this work, due to the large number of compounds to be extracted from a complex matrix such as mussels, several extraction parameters like pre-cleaning with n-hexane, acidification of the MeCN, and different types of extractive solvents, have been initially tested in order to assure the reliable extraction of musk and UV filters compounds. Then, optimized method was validated and applied in the assessment of musk and UV filter compounds in wild mussels collected in Portugal.

Quality assurance

Considering the widespread use of PCPs at high concentrations in a diversity of products, laboratory contamination can be a common situation. Precautions must be taken to prevent contamination from personnel, equipment and glassware. The use of any PCPs during either sampling or analytical procedures was avoided. Plastic material was avoided and all the glassware was previously rinsed with acetone before use.

The following optimization deals with the evaluation of the applicability of a previous QuEChERS and DLLME methodology, developed by Cunha *et al*. (2012) (116), to this kind of sample matrix (mussel) and to a large number of different chemical compounds (musk fragrances and UV filters).

Briefly, 0.5 g of freeze-dried homogenized sample was weight into a 40 mL dark glass tube and hydrated with 5 mL of deionized water. To QuEChERS procedure, 4 mL of acetonitrile (MeCN) was added and the tube was once again vortexed for 20 s. Next, the

salt mixture (2 g of anhydrous $MgSO_4$ and 0.5 g of NaCl) was added and the tube was placed on a wrist action shaker vortex for 30 s and taken to a round shaker for 30 min. Then it was centrifuged for 5 min at 3500 g. A 3 mL aliquot of the MeCN layer was transferred to a 4 mL glass tube, and 50 µl of the IS1 (Chrysene–d12, 3 mg/L) was added. The extract obtained by the QuEChERS procedure was used as the dispersive solvent for DLLME. So, 1 mL of this extract was transferred to a new vial and 50 µl of BP-d10 (IS2, 1 mg/L) and 60 µl of $C_2HCl_3$ were added and rapidly transferred to a 25-mL screw cap glass tube with conical bottom containing 3 mL of deionized water acidified to pH2, manually shaken and centrifuged for 3 min at 3500 g. The settled volume (~60 µl) was transferred to an insert, placed inside an injection vial and a volume of 1 µl was injected in the GC–MS system.

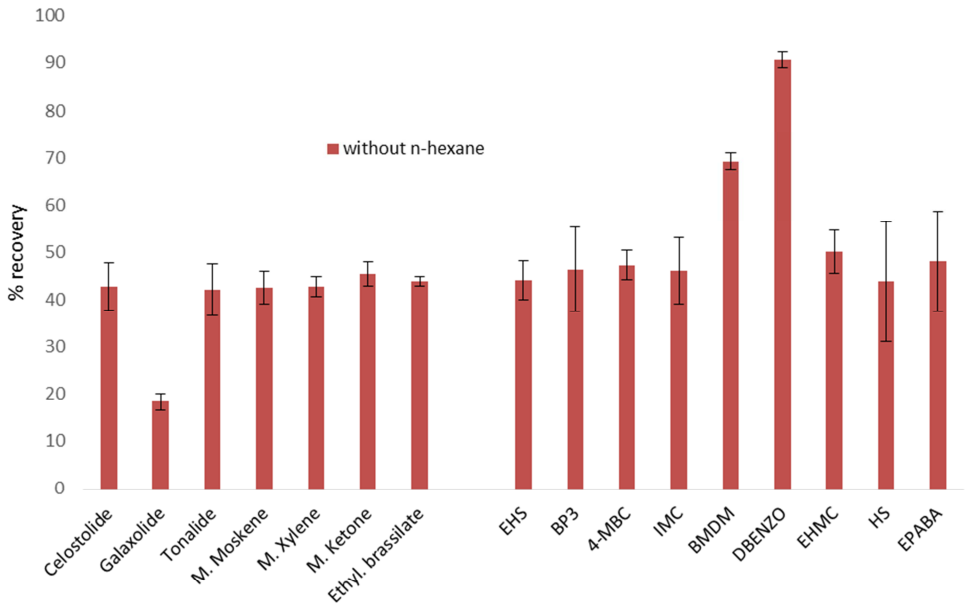



**Fig. 8 –** Recoveries (%) obtained for the different compounds using the extraction procedure based on Cunha *et al*. (2012) (116).

The % of recovery was determined by comparing the analytical response of the analytes in spiked samples with a mix of musks and UV filters at 1 mg/L each before and after QuEChERS. Recovery values obtained in this first approach were not very high, ranging from 18-46% for musks and from 44-91% for UV filters (Fig. 8). This may be due to the fact that mussels contain a considerable lipid portion (about 15%) and although fats are not very soluble in MeCN, a certain quantity of them can be co-extracted (117) interfering with the analytical response.

Improvements to overcome these results were designed as described in the following sections.

Sample pre-treatment

Knowing that high fat extracts can cause interferences in GC analysis and damage the GC equipment (column, liner, etc.) and in order to improve the recovery values obtained, a washing step with n-hexane was introduced before the QuEChERS-DLLME methodology described in the section above.

So, in order to evaluate the effect of n-hexane precleaning an experiment was conducted. Three sets of samples (A, B and C) were prepared as following: both A and B were initially spiked with a standards mixture (1 mg/L), while C was spiked with same mixture of standards in the end of QuEChERS.

In experiment A was added 2 mL of n-hexane before QuEChERS. In experiment B and C was performed QueChERS as described in previous section. All the experiments were subject to DLLME after QueChERS. The % of recovery was determined by comparing the analytical response of the analytes in spiked samples with a mix of musks and UV filters at 1 mg/L before (A and B) and after QuEChERS (C).

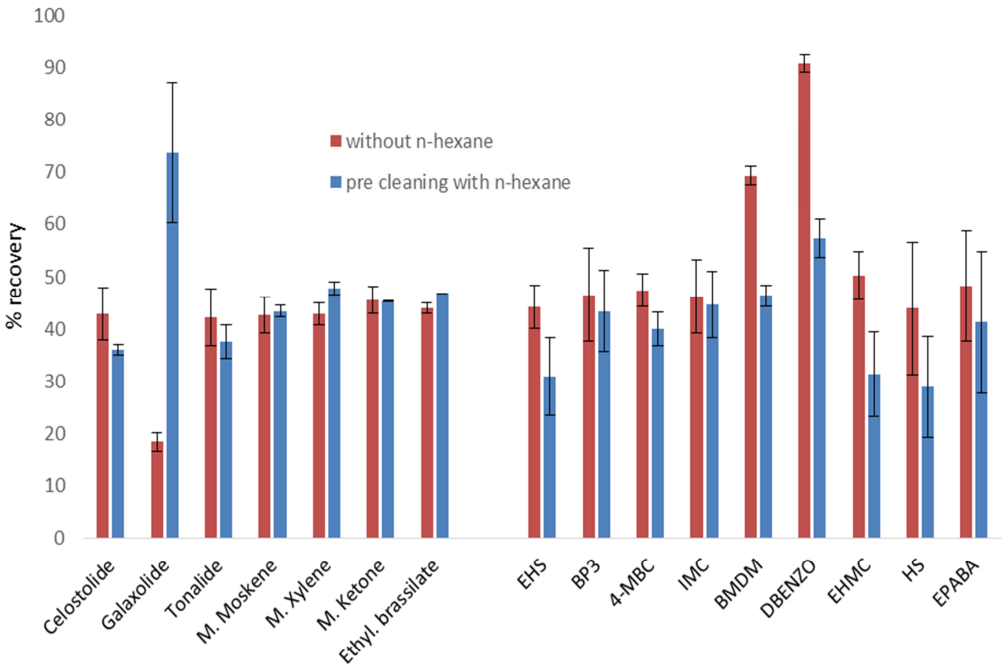



**Fig. 9 -** Effect of n-hexane pre-cleaning on the % of recovery of the target analytes.

Among musks, the use of n-hexane showed a slight improvement of the recoveries for the nitro musk xylene, from 43 to 48%, for the macrocyclic musk ethylene brassylate, from 44 to 47% and a more impressive improvement for the polycyclic musk galaxolide, from 18 to 74% (Fig. 9). It is worth noting that this value has an error associated of about 20% (RSD %). On the other hand, n-hexane pre cleaning impact on the recoveries of the UV filters was negative for all the compounds, especially for DBENZO; BMDBM, EHMC, HS and EHS, which showed decreases of 34, 23, 19, 15, and 13%, respectively.

This pre-cleaning step with n-hexane showed other weaknesses as increased time of analysis and difficulties to remove the n-hexane layer that produced a gel after the washing procedure, implicating extra centrifugation steps in order to make possible its complete remotion without loss of the compounds. So the optimization efforts were continued without this step.

The effect of MeCN acidification was further evaluated, taking in account that the maintenance of the better pH conditions throughout the process is important in order to ensure method reproducibility and improve the yields of extraction (57).

Acidification of the MeCN

Commonly, pH of the medium is an important parameter to consider in extraction procedures because it can affect the existing forms of some analytes in solution. This is special important in this case taking into account that some of the compounds in this study presented low pKa values, as can be seen in Table 4, so they are in ionized form at neutral pH, and it is well known, that this kind of analytes can be better extracted by organic solvents when they are in their neutral forms.

To investigate the effect of pH on extraction efficiency, MeCN was acidified to pH 2 by adding a few drops of 6N HCl solution. At lower pH the analytes exist mostly in their neutral forms being the ionization suppressed, which is beneficial for their transfer to the organic phase. At higher pH values the analytes undergo ionization, resulting in decreases extraction yields (57). The effect of MeCN acidification to pH 2 was evaluated considering the % of recovery, calculated as referred previously, for the selected analytes being the results presented in Table 13.

Slightly higher recoveries for almost analytes were achieved with the acidification of the MeCN to pH 2, as it was already reported by Cunha *et al*. (2015) (57). Increases in recoveries above 10% were observed for all the musks, being the most relevant for

galaxolide (36%) from 18 to 54%. Between the nitro musks, the increases in recoveries varied from 9%, 10% and 13% for M. ketone, M. moskene and M. xylene, respectively (Table 13). Similar recoveries improvements were achieved for some UV filters like EHS, BP-3, 4-MBC, IMC and HS (increases between 6-9%). However, for DBENZO, BMDM and EPABA, reductions of 4, 21 and 45% in the recovery was observed, probably because these compounds have lower pKa e.g. 2.39 for EPABA or some kind of degradation can occur.

**Table 13 –** Comparison of the recovery values (%) obtained with the acidification of the MeCN to pH 2.

| Family/compound | | MeCN | | MeCN pH2 | |
|---|---|---|---|---|---|
| | | Recovery (%) | RSD (%) | Recovery (%) | RSD (%) |
| *Musks* | **Celestolide** | 43 | 5 | 56 | 1 |
| | **Galaxolide** | 18 | 2 | 54 | 13 |
| | **Tonalide** | 42 | 5 | 54 | 3 |
| | **M. moskene** | 43 | 3 | 53 | 1 |
| | **M. xylene** | 43 | 2 | 56 | 1 |
| | **M. ketone** | 46 | 3 | 55 | 0 |
| | **Ethylene brassylate** | 44 | 1 | 62 | 0 |
| *UV filters* | **EHS** | 44 | 4 | 53 | 7 |
| | **BP-3** | 46 | 9 | 52 | 8 |
| | **4-MBC** | 47 | 3 | 54 | 3 |
| | **IMC** | 46 | 7 | 52 | 6 |
| | **BMDBM** | 69 | 2 | 48 | 2 |
| | **DBENZO** | 80 | 2 | 46 | 4 |
| | **HS** | 44 | 5 | 53 | 8 |
| | **EPABA** | 48 | 13 | 51 | 10 |
| | **EHMC** | 50 | 11 | 46 | 13 |

In view of these results, the MeCN acidification to pH 2 was adopted in the present QuEChERS methodology. As already referred, the MeCN extract resultant from the optimized QuEChERS will be used as dispersive solvent in the following DLLME procedure, being the optimization procedure of the DLLME further focus in the type of the extractive solvent.

Influence of the extractive solvent in DLLME procedure.

After QuEChERS, a DLLME procedure was performed to concentrate the compounds prior to their determination by GC. This approach allows the rapid extraction of lipophilic compounds from aqueous solutions by addition of an extractive and a dispersive solvent. The first is a high-density, water-insoluble solvent; whereas, acetone, methanol or acetonitrile are normally used as dispersive solvents. When this mixture comes in contact with the water a cloudy stage, consisting of fine particles of the extractor solvent dispersed into the aqueous phase, is formed. After centrifugation, the high-density solvent settles at the bottom of the extraction tube. Then, a fraction of the sedimented phase is injected in the chromatographic system (57). This technique uses a very small volume of extraction solvent and the contact surface between phases is infinitely large, leading to high enrichment factors and low extraction times. Rapidity, simplicity, low cost, effectiveness and high enrichment factors are the main advantages of this eco-friendly technique (57).

In DLLME, the equilibrium is achieved in few seconds due to the large contact surface between tiny droplets formed and the sample. Therefore, the mass transfer of the analytes from aqueous matrix to the extraction solvent was quickly realized. In short, DLLME can be regarded as a time-independent method (116).

One of the parameters that can be optimized in DLLME is the type of the extractive solvent, which should satisfy some requirements: (i) higher density than water, (ii) immiscibility with water, (iii) good extraction capability of the analyte(s), and (iv) chromatographic compatibility (57).

The experiments were carried out using trichloroethylene ($C_2HCl_3$) or carbon disulfide ($CS_2$) as extractive solvents. $C_2HCl_3$ has a density of 1.46 and its water solubility is 1.28 g/L at 25 °C, fulfilling the requirements previously described for a good extractive solvent. $CS_2$ has even lower solubility in water (2.17 g/L at 25°C) than $C_2HCl_3$ and its density is 1.26, being also a good candidate to be used as extractive solvent in the DLLME procedure. The extract obtained by QuEChERS was used as the dispersive solvent for DLLME, by transferring 1 mL of the MeCN extract to a 3 mL vial tube. After adding 50 µL of BP-d10 (IS2, 1 mg/mL), 60 µl of $C_2HCl_3$ or 80 µl of $CS_2$ were added to different extracts. Then, the mixture was rapidly transferred to a 25 mL glass tube with conical bottom containing 3 mL of deionized water acidified at pH 2. The tube was sealed and gently shaked by hand for 20 s and centrifuged at 3500 g for 3 min. The settled volume (~60 µl) was transferred to an insert, placed inside an injection vial and a volume of 1 µl was injected in the GC–MS system.

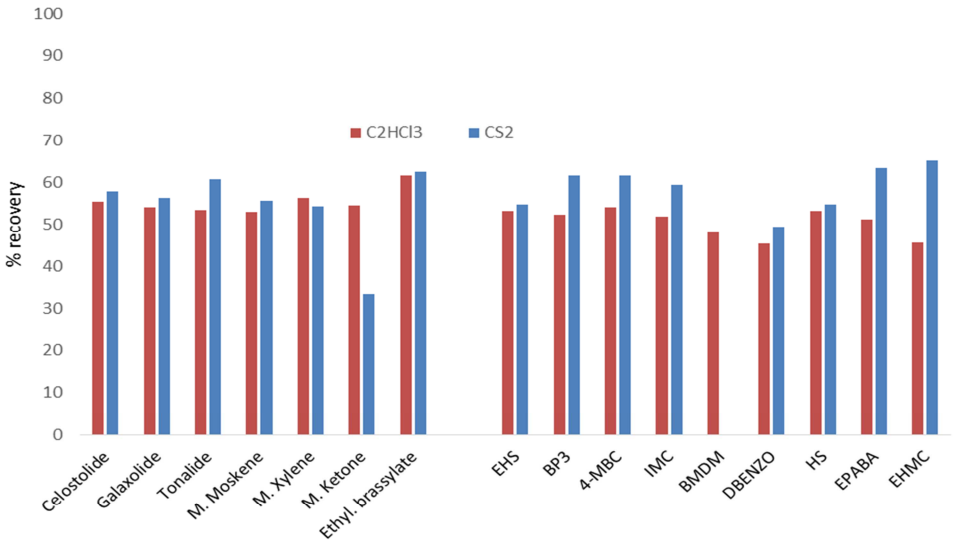



**Fig. 10 -** Recoveries of the analytes with 2 different extractive solvents: trichloroethylene ($C_2HCl_3$) and carbon disulfide ($CS_2$).

$CS_2$ used as extractive solvent in this DLLME experiments provided better recoveries than $C_2HCl_3$, especially for UV filters with increases in recoveries up to 20% (EHMC) and around 10% for BP-3, 4-MBC, EPABA and IMC. A similar effect of $CS_2$ improvement in recoveries was not observed in musk fragrances with the exception for tonalide, for which 8% increase was noticed. M. ketone showed a decrease of about 20% in the recovery with $CS_2$ as extractive solvent when compared to $C_2HCl_3$ and M. xylene a decrease around 2%. Despite the slight better recoveries with $CS_2$ when compared to $C_2HCl_3$ (Fig. 10), $CS_2$ presents some characteristics such as high toxicity, high volatility and instability, which lead us to choose $C_2HCl_3$ as extractive solvent to DLLME procedure.

Derivatization Agents

Taking into account the diversity of the compounds under study and in order to improve both the selectivity of the analysis and the efficiency of the chromatography, a derivatization step was attempted following the QuEChERS-DLLME extraction. Notwithstanding, it is important to note that most of these compounds, namely IMC, 4-MBC, EPABA, BMDBM and all the musk fragrances could be detected without derivatization (57).

Three different conditions were evaluated: i) no derivatization, ii) derivatization with a silylation reagent mixture (BSTFA with 1 % TMCS) and iii) derivatization with an acetylation reagent (acetic anhydride - AA).

A significantly higher intensity of the chromatographic response for the majority of the compounds was achieved with no derivatization process (Fig. 11). Only EHMC, galaxolide and EHS showed better recoveries when derivatized with AA and BSTFA, respectively. As a result, it was decided to choose to analyze the compounds without derivatization step.

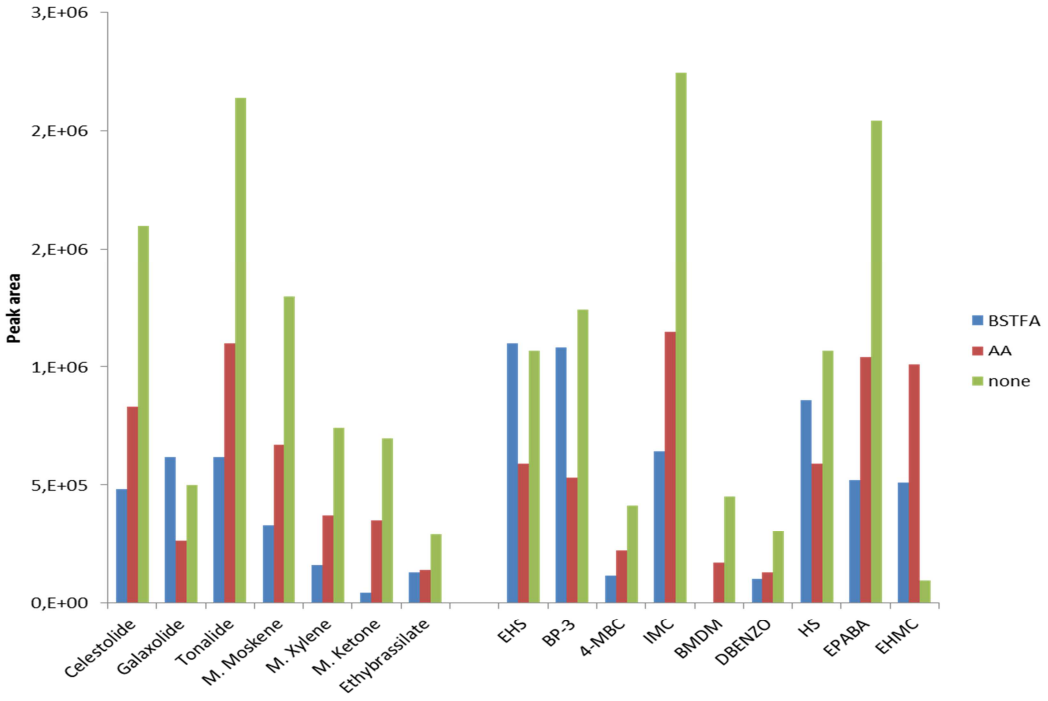



**Fig. 11 -** Results obtained using: no derivatization process, a silylation reagent mixture (BSTFA with 1 % TMCS) and an acetylation reagent (acetic anhydride – AA).

In view of the results obtained during the experiments to best adapt the QuEChERS-DLLME procedure previous developed by Cunha et al. (2012) (116), the best conditions were established as following: Pre-treatment - 0.5 g of freeze-dried homogenized sample, 5 mL of deionized water; QuEChERS - 4 mL of acetonitrile (MeCN), 2 g of anhydrous $MgSO_4$ and 0.5 g of NaCl, shaken for 1 h followed by centrifugation (5 min at 3500 g) – MeCN extract. DLLME – Transference of 1 mL of this extract to a new vial, addition of 60µl $C_2HCl_3$, Rapidly transference to a 25-mL screw cap glass tube with conical bottom containing 3 mL of deionized water acidified to pH 2, centrifugation (3 min at 3500 g). The settled volume (~60 µl) was transferred to an insert, placed inside an injection vial and a

volume of 1 µl was injected in the GC–MS system. A simplified scheme of the procedure is shown in Fig. 12.

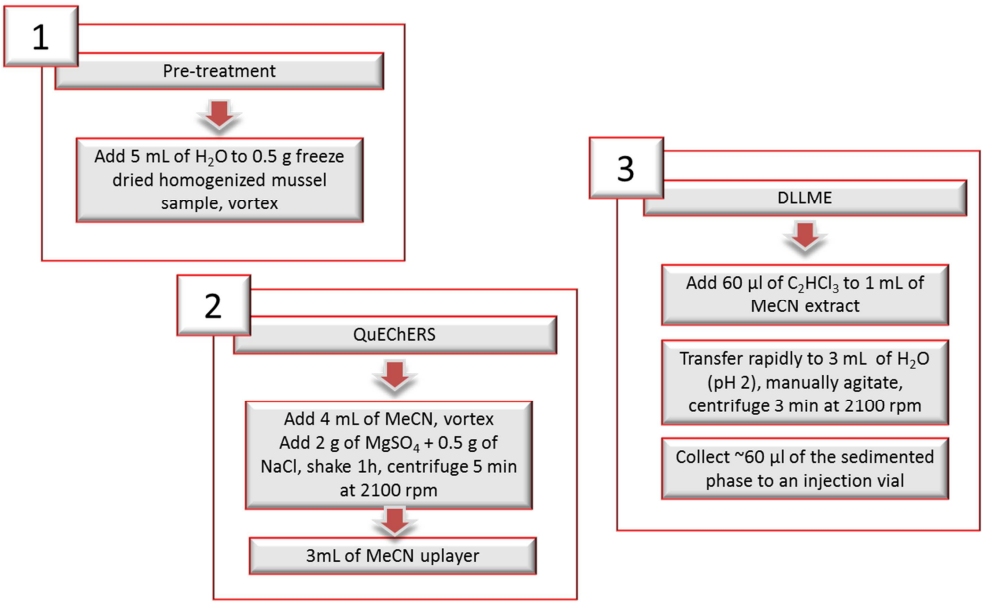



**Fig. 12 –** Flow chart of the sample preparation used in this study.

At this point, this combination of QuEChERS-DLLME showed to be an adequate, simple and fast extraction method for the detection of musk fragrances and UV filters from mussel samples, providing recoveries between 53-62% for the musk fragrances and 46-54% to UV filters, with the consumption of very low volumes of organic solvents, maintaining the important feature of eco-friendly procedure. Overall, is an inexpensive way to obtain a high enrichment factor and consequently good detection limits.

### 3.2. Method performance

After the adaptation of the QuEChERS-DLLME method, developed by Cunha *et al*. (2012) (116), to the detection of musk fragrances and UV filter compounds in mussel samples, the validation of the final developed procedure was needed in order to provide its application in the monitoring of coastal contaminants in real mussel samples. The validation process of the QuEChERS-DLLME-GC/MS method was carried out following the SANCO guidelines (118). The results are showed in Table 15.

Matrix effect

Initially, in order to evaluate the matrix effect, slopes of the calibration curves obtained from solvent ($H_2O$) and from matrix (standards added to mussel samples commercially acquired) were compared (Table 14), being observed a matrix suppression effect, as previously referred in other studies (11, 57, 119).

**Table 14 -** Results of the slopes obtained from the calibration curves conducted in $H_2O$ and in mussel.

| Family/compound | | Solvent ($H_2O$) | | Matrix | |
|---|---|---|---|---|---|
| | | CC slope | $r^2$ | CC slope | $r^2$ |
| *Musks* | **Celestolide** | **0.0395** | 0.976 | **0.0278** | 0.988 |
| | **Galaxolide** | **0.0136** | 0.982 | **0.0084** | 0.992 |
| | **Tonalide** | **0.0520** | 0.997 | **0.0352** | 0.996 |
| | **M. moskene** | **0.0300** | 0.989 | **0.0214** | 0.996 |
| | **M. xylene** | **0.0168** | 0.989 | **0.0125** | 0.997 |
| | **M. ketone** | **0.0172** | 0.981 | **0.0130** | 0.997 |
| | **Ethylene brassylate** | **0.0074** | 0.997 | **0.0048** | 1.000 |
| *UV filters* | **EHS** | **0.0306** | 0.983 | **0.0193** | 0.981 |
| | **BP-3** | **0.0517** | 0.964 | **0.0276** | 1.000 |
| | **4-MBC** | **0.0158** | 0.989 | **0.0095** | 0.993 |
| | **IMC** | **0.0376** | 0.997 | **0.0240** | 0.996 |
| | **BMDBM** | **0.0012** | 0.357 | **0.0055** | 0.982 |
| | **DBENZO** | **0.0003** | 0.198 | **0.0030** | 0.996 |
| | **EHMC** | **0.0609** | 0.975 | **0.0357** | 0.999 |

One of the major drawbacks in the analysis of biological samples is often the high matrix effect (ME) observed. Although GC–MS is a powerful instrumental technique, it is likely the observance of matrix effects, which may negatively affect the quantification of the target analytes. Therefore, in this study the percentage of matrix effect was calculated for each compound by the ratio of the slopes of the calibration curves (CC) obtained in matrix (mussel samples) and in the solvent ($H_2O$), which was then multiplied by 100 to get the enhancement or suppression in percentage (Eq. 1).

Eq.1:

$$\text{Matrix effect (\%)} = \frac{\text{m (CC matrix)}}{\text{m (CC H2O)}} \times 100$$

The value of 100% indicates that there are no significant ME; values above 100% signal signifies enhancement and values below 100% signal suppression (120). A signal suppression was observed for all the compounds in study (Fig. 13). Musk fragrances showed ME values ranging from 62 and 76% and slightly lower values for the UV filters (53-68%).

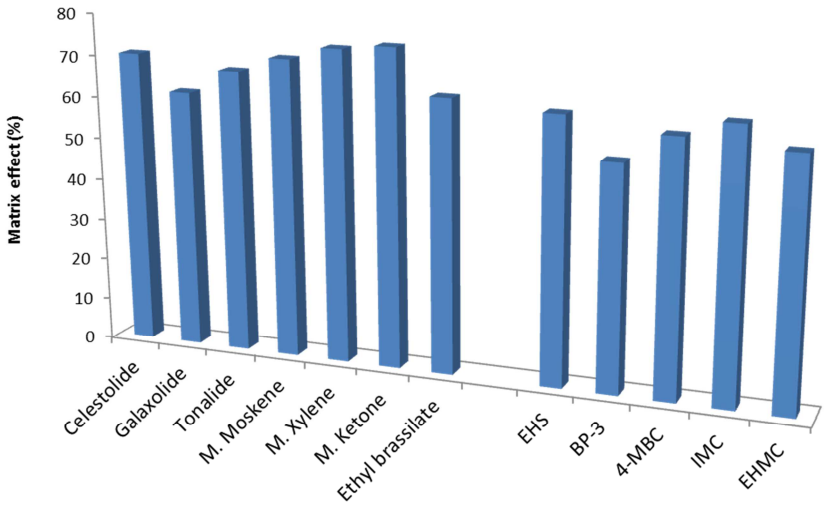



**Fig. 13** – Matrix effect (%) results for the selected analytes.

Taking in consideration the ME values obtained all the validation studies were performed by using mussel extracts obtained from samples free of the analytes of interest. The samples purchased from a local supermarket were freeze-dried and liofilized previously of being homogenized and stored at 4 °C, protected from light.

Linearity

Linearity was studied using matrix-matched calibration by analyzing the samples commercially acquired, spiked at nine concentration levels (0.5, 2, 5, 10, 50, 100, 250, 500 and 750 ng/g). All matrix-matched standard solutions used throughout the study were prepared by spiking 0.5 g of sample before QuEChERS with 50 µl of appropriate standard musk and UV filter solutions, prepared in MeCN. Spiked samples were left to stand for 1 h prior to extraction to allow compounds diffusion onto the matrix and evaporation of the solvent, and were extracted by the combined QuEChERS-DLLME method described in

the sample preparation section (Fig. 13). Figure 14 shows a total ion chromatogram of a mussel sample spiked with a mixture of musks and UV filters at 500 ng/g.

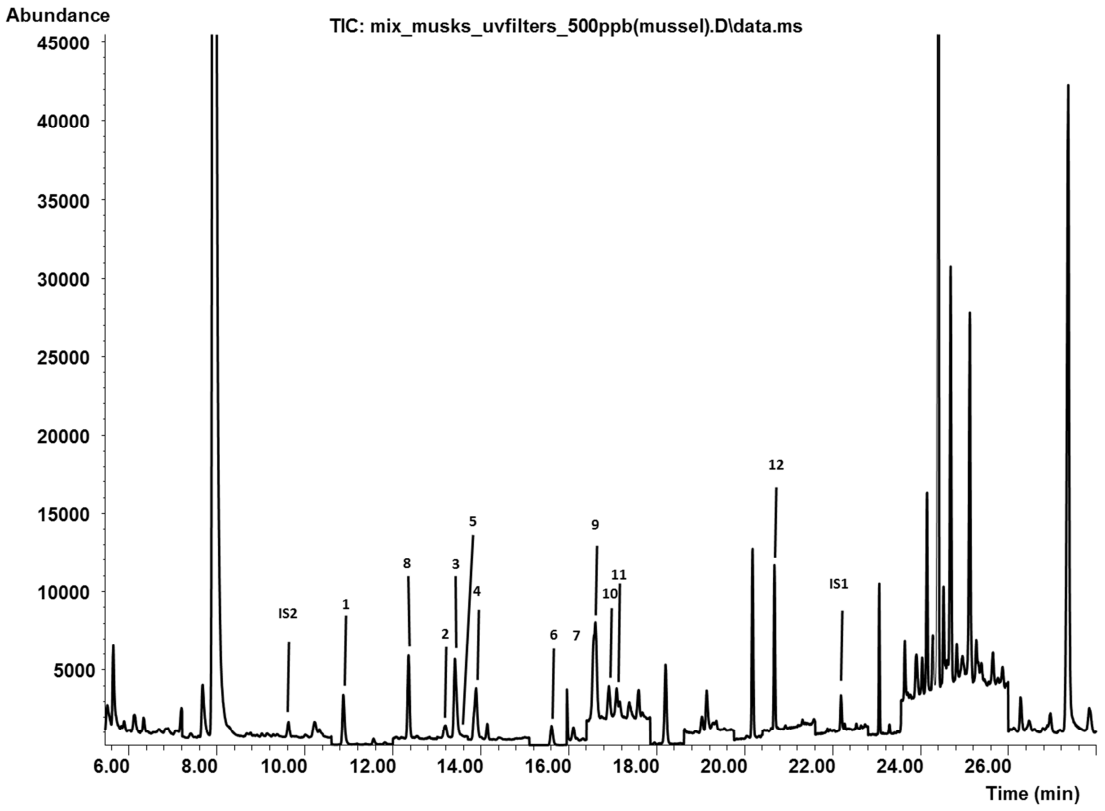



**Fig. 14** – Total ion chromatogram obtained with a mussel sample spiked with a mixture of musks (1 - celestolide, 2 - galaxolide, 3 - tonalide, 4 - M. moskene, 5 - M. xylene, 6 - M. ketone, 7 - ethylene brassylate) and UV filters (8 - EHS, 9 - BP-3, 10 - 4-MBC, 11 - IMC and 12 – EHMC) at 500 ng/g, and IS1 (Chrysene d-12, 3 mg/L) and IS2 (BP d-10, 1 mg/L).

Calibration curves were constructed by plotting the analytes/IS1 ratio obtained against the concentration of analytes. The IS1 (chrysene–d12; 3 mg/L), was added before QuEChERS extraction to keep track of possible losses occurring during the sample preparation. A second IS (IS2, BP-d10, 1 mg/L) was added before DLLME extraction, with internal quality control purposes, to account for possible losses during DLLME step and to monitorize GC injection. The results obtained demonstrated a good linearity within the tested interval, with correlation coefficients (r) always higher than 0.995 for all analytes (Table 15), provided by the presence of the IS1.

Limits of detection and limits of quantification

The quantification limits were established as the lowest concentration assayed quantified with acceptable accuracy and precision (RSD<15%), which were the lowest calibration level of the calibration curve. LOQ varied between 0.5 to 50 ng/g (dw) for the musk fragrances and from 0.5 and 100 ng/g (dw) for UV filters.

As it can be seen in Table 15, the lowest LOQs obtained for musks were 0.5 ng/g (musk moskene), 2 ng/g (celestolide) and 5 ng/g (dw) for musk ketone and galaxolide. Higher LOQs (50 ng/g (dw)) for musk xylene and ethylene brassylate were obtained. It was not possible to differentiate the responses among musks, in nitro and polycyclic, as it was for Cunha *et al*. (2015) (57) and Vallecillos *et al*. (2015) (70), in which the higher LOQs were obtained for the nitro musks and lower LOQs for polycyclic (2.5-7.5 ng/g (dw)). Picot Groz *et al*. (2014) (11) also reported high LOQ for musk ketone (50 ng/g). Even though, for the polycyclic musks, similar LOQs to those observed in this study, were reported (0.5 and 2.5 ng/g (dw) for galaxolide and celestolide, respectively). It's worth noting that the results in the different studies mentioned earlier, were obtained in mussels using QuEChERS. Among UV filters, EHMC and BMDBM presented the lowest LOQs of 0.5 ng/g (dw), and DBENZO and HS the highest with 100 ng/g (dw). These are similar to those reported by Picot Groz *et al*. (2014) (11) and Bachelot *et al*. (2012) (56) (5 ng/g for EHMC) in mussels using QuEChERS and MAE followed by GC/MS and RP-HPLC, respectively.

The method detection limits (LOD) were determined by successive analysis of diluted extracts until a 3:1 signal-to-noise ratio (S/N) was reached (121). Lower LODs were achieved in this study for the nitro musk moskene 0.5 ng/g (dw) and similar value for musk ketone with 5 ng/g (dw), when compared to those obtained by Cunha *et al*. (2015) (57). Musk xylene showed a higher LOD of 50 ng/g (dw) 10 times higher to that reported by Cunha *et al*. (2015) (57).

Recovery

For the recovery studies, samples were spiked with 50 µl of mixed standard solutions of the compounds; the final extract (3 mL) was placed into vials and spiked with 50 µl of the internal standard BP-d10 (IS2, 1 mg/L). Three spiking levels (50, 100 and 500 ng/g) were selected and six replicates (n=6) analysed at each level. Peak areas of analytes in samples spiked before QuEChERS were compared with peak areas obtained from similar samples extracted containing IS1 and analytes at same levels, with the standards addition

done after extraction. Good recoveries (between 62% and 77%) were obtained for the 2 higher concentration levels (100 ng/g and 500 ng/g (dw)). Worse results were obtained for the recoveries determined at 50 ng/g level, with recoveries comprised between 30% and 159%. These results are similar to those obtained by Saraiva *et al*. (2016) (69) who observed the same trend, using QuEChERS followed by GC/MS.

Precision

Intra-day and inter-day precision were evaluated at 100 ng/g level. For that purpose, six spiked samples were extracted and analysed in two different days for a period of three weeks. The presence of the IS improved the method repeatability, expressed as % of relative standard deviations (%RSD) with precision values below 18% for all compounds at the three concentrations levels. Inter-day repeatability values achieved (n=6, 100 ng/g (dw)) were below 15% (Table 15).

**Table 15-** Performance of the analytical protocol: recovery (%) and repeatability (%RSD), for the compounds in study, obtained in mussel spiked samples using QuEChERS followed by DLLME and GC-MS analysis (n=6).

| Family/compound | | Linearity | LOD | LOQ | Recovery (%) | | | Intra-day precision RSD (%) | | | Inter-day precision RSD (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $r^2$ | (ng/g) | (ng/g) | 50 ng/g | 100 ng/g | 500 ng/g | 50 ng/g | 100 ng/g | 500 ng/g | 100 ng/g |
| *Musks* | **Celestolide** | 0.998 | 2 | 2 | 55 | 76 | 66 | 5 | 10 | 10 | 2 |
| | **Galaxolide** | 0.998 | 5 | 5 | 74 | 72 | 65 | 9 | 6 | 10 | 11 |
| | **Tonalide** | 0.996 | 0.5 | 10 | 72 | 76 | 64 | 3 | 10 | 9 | 7 |
| | **M. Moskene** | 0.998 | 0.5 | 0.5 | 51 | 72 | 64 | 5 | 13 | 9 | 5 |
| | **M. Xylene** | 0.995 | 5 | 10 | 64 | 74 | 62 | 18 | 10 | 9 | 9 |
| | **M. Ketone** | 0.994 | 5 | 5 | 30 | 73 | 73 | 7 | 11 | 10 | 7 |
| | **Ethylene brassylate** | 0.999 | 50 | 50 | - | 77 | 68 | - | 18 | 10 | 13 |
| *UV filters* | **EHS** | 0.997 | 0.5 | 5 | 96 | 82 | 74 | 7 | 8 | 9 | 7 |
| | **BP-3** | 0.994 | 2 | 2 | 107 | 67 | 62 | 4 | 9 | 9 | 11 |
| | **4-MBC** | 0.997 | 2 | 5 | 135 | 69 | 63 | 6 | 8 | 9 | 8 |
| | **IMC** | 0.998 | 5 | 5 | 83 | 69 | 69 | 4 | 9 | 9 | 11 |
| | **BMDM** | 0.990 | 0.5 | 0.5 | 30 | 40 | 46 | 9 | 15 | 3 | 15 |
| | **DBENZO** | 0.999 | 50 | 100 | 159 | 87 | 60 | 7 | 9 | 8 | 8 |
| | **HS** | 0.988 | 50 | 100 | - | 88 | 64 | 6 | 9 | 8 | 11 |
| | **EPABA** | 0.999 | 0.5 | 2 | 116 | 85 | 46 | 7 | 6 | 9 | 8 |
| | **EHMC** | 0.991 | 0.5 | 0.5 | 98 | 80 | 57 | 3 | 9 | 10 | 11 |

Notes: RSD - Relative standard deviation; LOD – Limit of detection; LOQ – Limit of quantification; (-) not determined.

### 3.3. Occurrence of Musk fragrances and UV filters in mussels samples

The optimized and validated QuEChERS-DLLME GC-MS method was applied to quantify musk and UV filter compounds in mussel samples collected along Portuguese shores, at seven different locations (Viana do Castelo, Leça da Palmeira, Vagueira, Algés, Costa da Caparica, Aljezur and Faro) in five sampling campaigns (January, March, May, July and October), during 2015, resulting in 30 composed samples. Results are presented on Table 16.

In Figure 15 is showed a positive sample collected in Algés, (October of 2015) obtained by the optimized QuEChERS-DLLME-GC-MS method, together with the individual chromatogram in selected ion monitoring (SIM) mode of tonalide and BP-3.

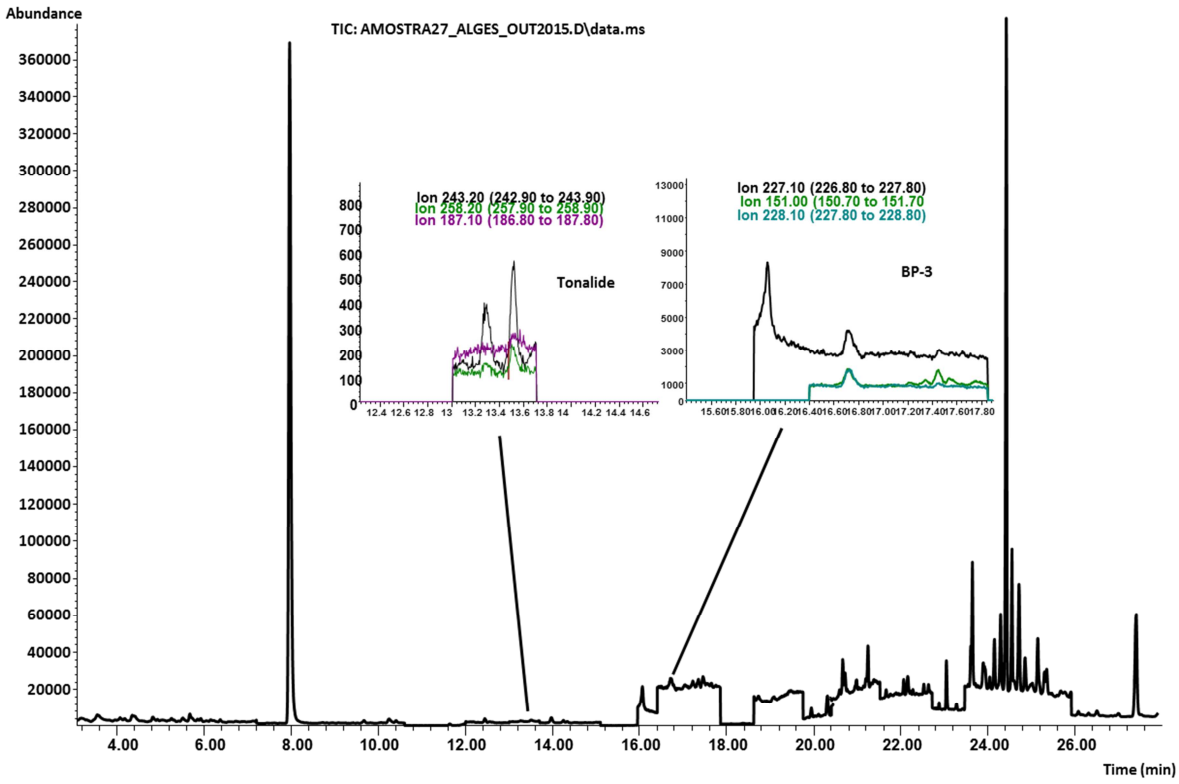



**Fig. 15** – Total ion chromatogram (TIC) of mussel sample of October (2015) from Algés obtained by the optimized QuEChERS-DLLME-GC-MS method, together with the individual chromatogram in selected ion monitoring (SIM) mode of tonalide and BP-3.

*Musk fragrances in mussels*

Representing two classes of PCPs, a total of twelve compounds, seven musk fragrances and five UV filters, were evaluated in this work (Table 16). Two of the seven musks, ethylene brassylate and M. ketone were not detected in any sample. Ethylene brassylate, a macrocyclic musk pertaining to the third generation of synthetic musks, is reported to be used in much less extent in cosmetic products because of its high production costs, so it was expected to be less bioaccumulated than the others (122). No detected levels of M. ketone were also reported by Cunha *et al*. (2015) (57) and Vallecillos *et al*. (2015) in mussels and fishes collected in European coast.

M. xylene (nitro musk) was detected in only one sample (January, Algés) at 18.4 ng/g (dw). In 2009, the International Fragrance Association (IFRA), a self-regulating system in the fragrance industry, voluntarily banned M. xylene because of its potential effects in the environment. The European Commission announced, in 2011, a decision to ban musk xylene under REACH (123). These two referred nitro musks (M. ketone and M. xylene) are listed in Annex III of EU Cosmetics Directive for restricted substances. M. xylene is provisionally allowed to be used up to 1.0% in fine fragrance, up to 0.4% in eau de toilette and up to 0.03 % in other cosmetic products, while for M. ketone the concentrations allowed are 1.4% in fine fragrance, up to 0.56% in eau de toilette and up to 0.042% in other cosmetic products (24). Despite this restrictions, low levels of M. xylene were detected in seafood, ranging from 0.013 ng/g (65) and <50 ng/g (dw) (11) along French and Portuguese coasts, respectively. The presence of M. ketone in mussels was observed by Saraiva *et al*. (2015) (65), below the LOQ (0.002 ng/g (dw)).

Interestingly, M. moskene was detected in six mussel samples in this study, wich correspond to 20% of detection frequency, although at low levels, ranging from 9.3 ng/g (dw) to 15.2 ng/g (dw). This is surprising taking into account that this compound is listed in Annex II of EU Cosmetics Directive for prohibited substances in cosmetic products, and it was phased out since the 80s (13). In the literature, no detected levels are reported for the occurrence of this compound in aquatic biota (57, 119) and most frequently, this compound is not even analysed. However, nitro musks are still being produced in China and India and used in non-cosmetic compounds, like detergents and household cleaning products in the USA (122), contributing to the levels found in environment.

Overall, given the environmental persistence and the continued use of these compounds even at low concentrations, attention should be given on nitro musks occurrence and the

possible toxicological effects by long term exposure, despite of their restricted or forbidden status.

For the polycyclic musks (celestolide, galaxolide and tonalide) a different trend was observed. Celestolide was detected in only one sample (14.5 ng/g (dw)) at Leça da Palmeira in March. Low detection frequency for celestolide, when compared with the other polycyclic musks, was also observed by Cunha *et al*. (2015) (57), Vallecillos *et al*. (2015) (70) and Saraiva *et al*. (2016) (65).

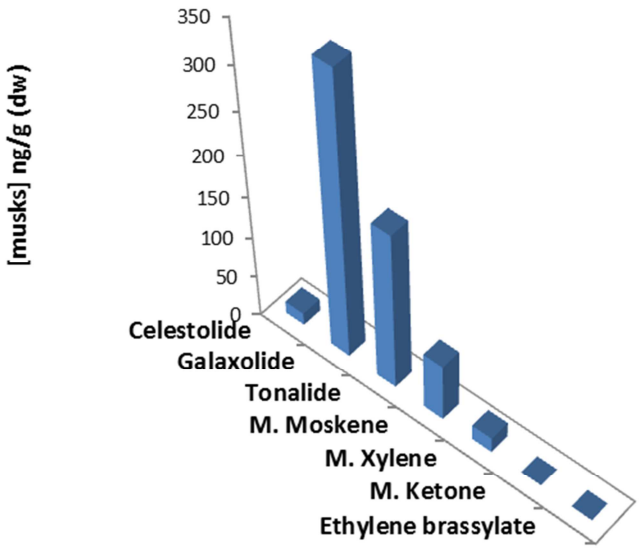



**Fig. 16** – Total concentrations (ng/g dw) observed for the different musks in the mussel samples analysed.

Galaxolide and tonalide are the most frequently used musks in cosmetics, being considered high volume production chemicals (HVPC). Galaxolide, despite having been detected in only 20% of the samples, reached one of the highest concentrations measured in this study, 159.4 ng/g (dw) at Costa da Caparica in January. Cunha *et al*. (2015) (57) found lower levels of galaxolide in mussels collected from Po estuary (34.52 ng/g) similar to those found in mussels by Ziarrusta *et al*. (2015) (66) in Central America coast (45 ng/g). However, higher levels of galaxolide were reported in mussels along Asia-Pacific coast ranging from 110 to 3300 ng/g (dw) (67).

Tonalide was the most frequently detected musk in this study (57%), with levels ranging from not detected (in twelve samples) up to 31.7 ng/g (dw), being worth noting that this maximum concentration was observed at Costa da Caparica in January, the same sample

where galaxolide reached its maximum concentration. Lower levels have been reported in mussels collected in European coast ranging from 1.23 to 12.99 ng/g (dw) (57, 65, 70). Nakata *et al*. (2012) (67) and Subedi *et al*. (2014) (19) reported levels ranging from not detected up to 860 ng/g (dw) in mussels collected in Asia Pacific coast and USA coast. A correlation between these two compounds has been described in literature (57), stating similar detection frequencies for both compounds and higher concentrations of galaxolide. This supports that galaxolide, as previously referred, is used and produced in a higher proportion than tonalide, wich translates in the detected levels in aquatic biota (11, 19, 26, 57, 67, 119). In our results galaxolide is also the most prevalent compound (Fig. 16), however a higher detection frequency for tonalide was observed. Nevertheless, in every sample where galaxolide was detected tonalide was also present at lower levels than the first (Table 16). Similar results were reported by Ziarrusta *et al*. (2015), Saraiva *et al*. (2016) and Reiner *et al*. (2011) (66, 69, 71).

**Table 16** – Musks and UV filters levels (ng/g dw) measured in 2015 sampling campaign in Portuguese beaches.

| Sampling | | Compounds | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Musks* | | | | | | | *UV filters* | | | | |
| Location | Date | **Celestolide** | **Galaxolide** | **Tonalide** | **M. Moskene** | **M. Xylene** | **M. Ketone** | **Ethyl brassylate** | **EHS** | **BP-3** | **4-MBC** | **IMC** | **EHMC** |
| Viana do Castelo | January | nd | nd | 23.3 | nd | nd | nd | nd | <5 | nd | nd | nd | nd |
| | March | nd | nd | <10 | nd | nd | nd | nd | 9.1 | nd | nd | nd | 51.3 |
| | May | nd | nd | <10 | nd | nd | nd | nd | 6.4 | nd | nd | nd | 4.1 |
| | July | - | - | - | - | - | - | - | - | - | - | - | - |
| | October | nd | nd | <10 | nd | nd | nd | nd | 25.4 | nd | nd | nd | <0.5 |
| Leça da Palmeira | January | nd | nd | <10 | 12.7 | nd | nd | nd | 25.6 | nd | nd | nd | nd |
| | March | 14.5 | nd | nd | nd | nd | nd | nd | 52.0 | nd | nd | nd | 75.0 |
| | May | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | 11.8 |
| | July | nd | 27.5 | <10 | nd | nd | nd | nd | 5.4 | <2 | nd | nd | 14.7 |
| | October | nd | nd | nd | nd | nd | nd | nd | nd | 622.1 | nd | nd | 69.8 |
| Vagueira | January | - | - | - | - | - | - | - | - | - | - | - | - |
| | March | - | - | - | - | - | - | - | - | - | - | - | - |
| | May | nd | nd | <10 | nd | nd | nd | nd | 17.9 | 51.6 | <50 | 24.9 | 49.8 |
| | July | nd | nd | nd | nd | nd | nd | nd | <5 | nd | nd | nd | nd |
| | October | nd | 15.6 | 17.5 | nd | nd | nd | nd | 38.9 | 106.9 | 74.6 | 40.4 | 67.0 |
| Algés | January | nd | 55.3 | 13.0 | 9.3 | 18.4 | nd | nd | 22.0 | 99.2 | 60.3 | 43.1 | nd |
| | March | nd | 46.3 | 12.7 | 9.6 | nd | nd | nd | 30.3 | 121.5 | 88.3 | 30.5 | 94.1 |
| | May | - | - | - | - | - | - | - | - | - | - | - | - |
| | July | nd | nd | nd | 10.5 | nd | nd | nd | 7.2 | nd | nd | nd | nd |
| | October | nd | 37.1 | 11.3 | nd | nd | nd | nd | 19.3 | 89.2 | 67.3 | 33.2 | 48.3 |

Notes: nd – not detected; (-) not determined.

**Table 16** – Musks and UV filters levels (ng/g dw) measured in 2015 sampling campaign in Portuguese beaches (cont.).

| Sampling | | Compounds | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Musks* | | | | | | | *UV filters* | | | | |
| Location | Date | **Celestolide** | **Galaxolide** | **Tonalide** | **M. Moskene** | **M. Xylene** | **M. Ketone** | **Ethyl brassylate** | **EHS** | **BP-3** | **4-MBC** | **IMC** | **EHMC** |
| Costa da Caparica | January | nd | 159.4 | 31.7 | nd | nd | nd | nd | nd | nd | nd | nd | 181.8 |
| | March | nd | nd | nd | 12.8 | nd | nd | nd | 24.0 | 51.2 | nd | nd | 69.6 |
| | May | nd | nd | nd | nd | nd | nd | nd | 59.3 | nd | nd | nd | <0.5 |
| | July | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| | October | nd | nd | nd | 15.2 | nd | nd | nd | nd | nd | nd | nd | nd |
| Aljezur | January | nd | nd | <10 | nd | nd | nd | nd | 5.6 | nd | nd | nd | <0.5 |
| | March | nd | nd | nd | nd | nd | nd | nd | 11.5 | nd | nd | nd | 26.2 |
| | May | nd | nd | nd | nd | nd | nd | nd | 11.6 | nd | nd | nd | <0.5 |
| | July | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| | October | nd | nd | nd | nd | nd | nd | nd | 7.0 | nd | nd | nd | <0.5 |
| Faro | January | nd | nd | 26.0 | nd | nd | nd | nd | nd | nd | nd | nd | <0.5 |
| | March | nd | nd | 18.7 | nd | nd | nd | nd | nd | nd | nd | nd | 34.9 |
| | May | nd | nd | 20.1 | nd | nd | nd | nd | 13.5 | nd | nd | nd | <0.5 |
| | July | - | - | - | - | - | - | - | - | - | - | - | - |
| | October | nd | nd | 18.3 | nd | nd | nd | nd | 9.6 | nd | nd | nd | <0.5 |

Notes: nd – not detected; (-) not determined.

*UV filters in mussels*

The UV filters detected in the sampling sites were quantified at higher concentrations and more frequently in mussel tissues than musk fragrances (Fig 17).

Both 4-MBC and IMC showed a detection frequency of 17%, interestingly being found in the same sampling points (Algés and Vagueira), with concentrations ranging from 40.8 to 88.3 ng/g (dw) and from 24.9 to 43.1 ng/g (dw), respectively. A high correlation with Spearman's rank correlation analysis was observed for these two compounds ($r=0.991$, $p \leq 0.001$), indicating a probable similar source. Recently, Gago-Ferrero *et al*. 2015 (3), reported lower levels for 4-MBC in aquatic biota, ranging from not detected up to 2.7 ng/g (dw). Much higher levels were reported by Balmer *et al*. 2005 (124) and Buser *et al*. 2006 (59) in fishes from lakes and rivers in Switzerland with high recreational activities, reaching maximum concentrations of 1800 ng/g (dw). However, Cunha *et al*. (2015) (57), analyzing UV filters in mussels collected from European coast, did not found detectable levels of these two compounds. It is worth noting that both 4-MBC and IMC are not allowed to use in cosmetics in USA and Japan, while in EU they can be incorporated in cosmetics in concentrations up to 4 and 10 %, respectively (125).

BP-3 was found in eight out of the 30 samples analysed, corresponding to 27% of detection frequency, reaching a maximum concentration of 622.1 ng/g (dw) at Leça da Palmeira in October. It was found in levels higher than 150 ng/g (dw) in other two locations, Algés and Vagueira (Fig. 14). Levels reported in literature for BP-3 in aquatic organisms, ranged from not detected to 123 ng/g (dw) in mussels and perchs from European coast and lakes in Switzerland, respectively (57, 124). Higher levels up to 1037 ng/g (lw) were detected in cod liver by Langford *et al*. 2015 (61).

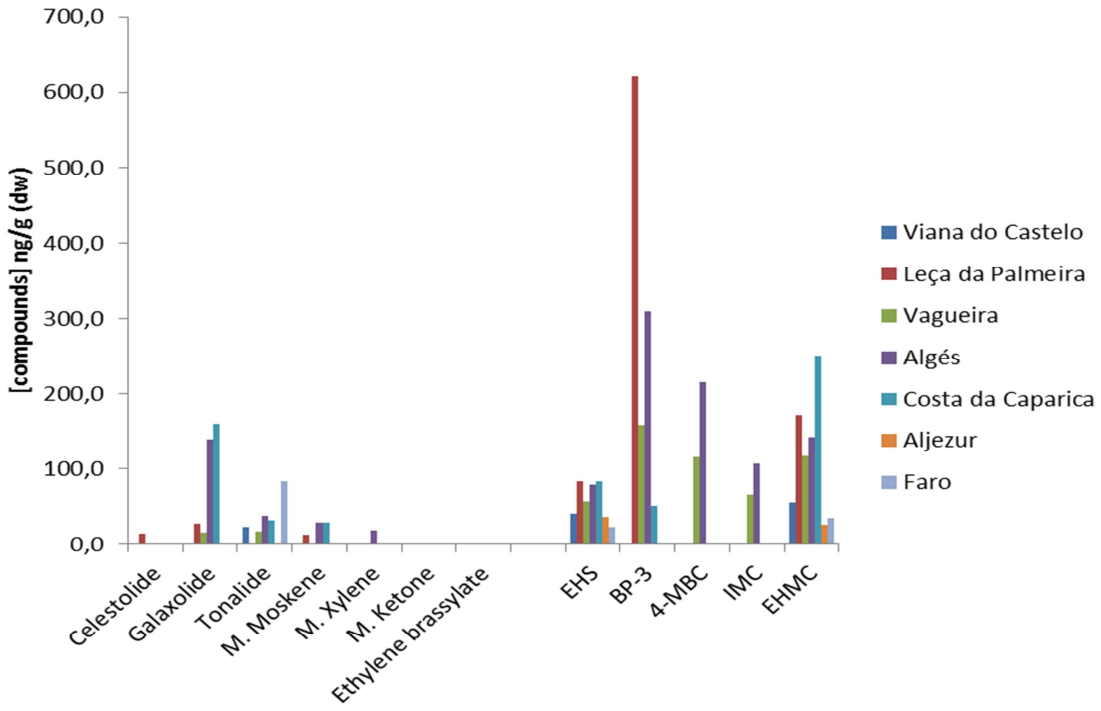



**Fig. 17** – Variation of the concentrations (ng/g dw) observed for musks and UV filters, in the mussel samples analysed, along the different sampling sites, during 2015.

EHS and EHMC both showed a detection frequency of 73%, however they were not significantly correlated (r=0.150, p≤0.001). Cunha *et al*. (2015) (57) reported not detected levels for EHS and below 20 ng/g (dw) for EHMC in mussels collected in European coast. Despite the relative high concentrations of 181.8 ng/g (dw) found in this work for EHMC, in Costa da Caparica (January), Picot Groz *et al*. (2014) (11) found levels up to 1765 ng/g (dw) in mussels collected in the south of Portugal. This may be explained by differences in the sampling conditions (location, time points, etc.). In a similar range than our results, levels of 240 ng/g (dw) were reported by Bachelot *et al*. (2012) (56) in mussels from French coasts.

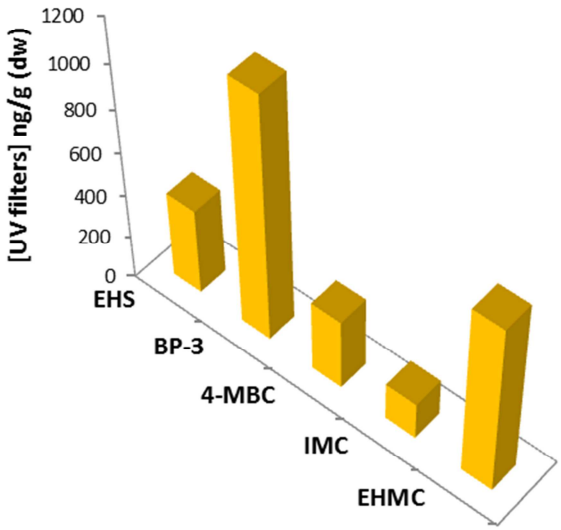



**Fig. 18** – Total concentrations (ng/g dw) observed for the different UV filters in the mussel samples analysed.

Overall, EHS was detected at lower total levels than EHMC, with maximum total concentrations of 401.8 and 798.4 ng/g (dw), respectively. An explanation can be that both EHS and EHMC are allowed to be use in cosmetics worldwide, but the maximum concentrations allowed are lower for EHS (5-10% and 7.5-20%, respectively) (125). BP-3 and EHMC showed the highest total concentrations for the UV filters analysed during the monitoring study in 2015 (Fig. 18) reaching total concentrations up to 1141.6 and 798.4 ng/g (dw), respectively. Apart from the fact that they are extensively used not only in several personal care products but also in food additives, plastics, detergents and paints, and may enter the environment directly or indirectly through wastewater, they both are allowed for use in cosmetics by all the different regulations worldwide at concentrations >10 % up to 20 % (125). For these reasons, its occurrence may be higher than the rest of the UV filters analysed. A significant correlation was observed for BP-3 and EHMC ($r=0.536$, $p\leq0.001$) indicating probable similar sources.

Geographical distribution

Seven locations along Portuguese shore were sampled during 2015, from January to October. Overall, UV filters were more frequently detected and quantified at higher concentrations in mussel than fragrances, as it was previously referred and as it can be seen in Figure 19.

Overall, Algés was the location which presented more incidences of the compounds analysed and showed the highest concentrations values for musks and UV filters in mussels, followed by Leça da Palmeira, Costa da Caparica and Vagueira. On the other hand, in Viana do Castelo and Faro fewer compounds were present. Low levels for UV filters (61.9 ng/g (dw) and not detected levels for musks were observed in Aljezur.

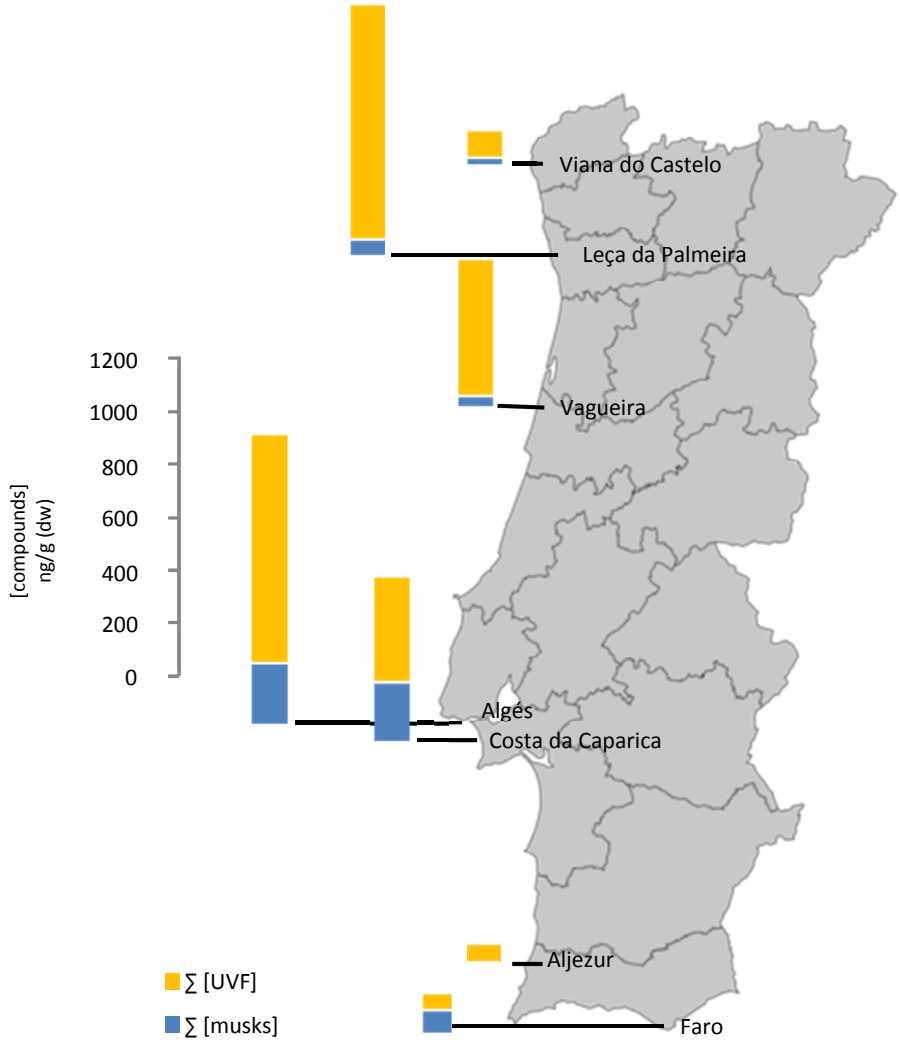



**Fig. 19** – Geographical distribution of the total concentrations (ng/g dw) for musks and UV filters, observed in mussel samples.

Algés beach is located in the mouth of Tagus estuary, right beside Pedrouços dock. The Tagus estuary lies in great Lisbon metropolitan area, with about 3.2 million inhabitants. It´s a region with high population density and intense industry and agriculture activities. The estuary receives discharges, many times without any treatment, from all the anthropogenized area surrounding it, ending all up near Algés beach. This human

pressure can be reflected by the high total levels of musks and UV filters detected in mussels collected from Algés beach (223.7 and 853.7 ng/g dw, respectively). Costa da Caparica beach is also located in Lisbon metropolitan area and not very far from Algés (~15 Km). Situated on the occidental coast of the Peninsula of Setúbal, the territory is the result of ocean receding, covering an area of 10.18 km² between the water and main escarpment. Its coastal extent represents the largest contiguous beach in Portugal, with an expanse of approximately 30 km. It is in a protected area called "Costa da Caparica Fossil Cliff Protected Landscape". The lower total levels found in this beach (385.9 and 219.2 ng/g (dw) or UV filters and musks, respectively) when compared with Algés could be not only for being a part of a protected area, but also to the fact of Costa da Caparica is facing the Atlantic Ocean. It is worth noting that the total musks concentrations observed were quiet similar for Algés and Costa da Caparica (223.7 and 219.2 ng/g (dw), respectively, illustrating their proximity and probable similar sources for this contaminants in the Tagus estuary area, being these the two locations with higher incidence of musks (Fig 19).

Leça da Palmeira beach is located in the mouth of Leça estuary, close to Leixões Harbor and Matosinhos refinery. Contrary to Tagus estuary, Leça is small and is very artificialized, being occupied almost entirely by the Leixões harbor, the largest artificial port in northern Portugal. It lies in Porto metropolitan area, with about 1.7 million of inhabitants, and receives all the discharges from the surrounding areas heavily industrialized (Maia and Matosinhos). Also, it is located near to Douro estuary, being this a strong contributor especially during winter season with winds coming from the south. Due to the strong anthropogenic pressure of the area, the Leça River has been considered by the Portuguese authorities as one of the most polluted aquatic environments in the North of Portugal and many efforts have been made to try to reverse this situation (126). The high total levels of UV filters detected in mussels collected in Leça da Palmeira beach (876.5 ng/g (dw)) probably illustrate the discharges of untreated wastewaters along the river as well as high human pressure with intense recreational activities like swimming and surfing. These levels were similar to those found in Algés (856.7 ng/g (dw)). Rocha *et al*. (2012) (126) found levels of endocrine disruptor compounds (ECDs) in seawater in Leça da Palmeira beach, up to 150 ng/L, alerting for the high contamination of this estuary. On the other hand, musk fragrances were detected at lower levels (54.8 ng/g (dw)). A similar trend was observed in Vagueira with high levels for UV filters (512.6 ng/h (dw)) and lower musks concentrations (33.1 ng/g (dw)). Vagueira beach, located near Aveiro lagoon and estuary, faces the Atlantic Ocean. Aveiro lagoon suffers from human pressures such as discharges from industrial point sources (main

source), disposal of solid waste management and leaching from agricultural areas, being covered by DPSIR (Driving forces and Pressures on an area, the State of the environment and the Impacts these forces have and the Responses that are undertaken) - a causal framework for describing the interactions between society and the environment – for assessing mercury pollution (127).

A different trend was observed for Viana do Castelo and Faro. Faro is located in the protected area of "Parque Natural da Ria Formosa". This situation as well as not being close to highly density populations could contributed for the low levels of musks and UV filters found in mussels collected in these beaches. Both musks and UV filters were detected in lower total levels when compared to the other sampling locations, and in the same range (119.6 and 141.2 ng/g (dw)). Despite the recreational activities from the bathers, in Viana do Castelo a total concentration of 96.3 ng/g (dw) for UV filters and 23.3 ng/g (dw) for musks were observed. Inversely, in Faro it was observed a higher total level for musks (83.1 ng/g (dw) corresponding only to tonalide) than for UV filters (58.1 ng/g (dw)).

Aljezur, located in "Parque Natural do Sudoeste Alentejano e Costa Vicentina", was the location with less incidence of the compounds. Musks were not detected and EHS and EHMC were the only UV filters detected at a total concentration of 61.9 ng/g (dw), similarly to Faro where UV filters detected were in the same range of concentrations and only EHS and EHMC were present.

At whole, a geographical variation was observed along the Portuguese coastal areas, being the higher levels related to the higher population density, the proximity to estuaries and industrial activities. Low levels were observed in the beaches located in protected areas and facing the Atlantic Ocean, with less population density.

Seasonal variation

A seasonal variation was observed being the highest total concentrations of musks and UV filters observed in October (1434.1 ng/g (dw)), followed by March (884.2 ng/g (dw) and January (786.7 ng/g (dw)). The lowest total concentrations of musks and UV filters were observed in July (65.4 ng/g (dw) followed by May (329.8 ng/g (dw)) as Figure 20 points out. From this data it is possible to infer that values are higher after the bathing season for the same location (Table 15). Similarly, Fent *et al*. (2010) (55) showed that fresh water mussels collected in a Swiss lake where bathing was practiced had higher concentrations after summer than before. These data are slightly distinct to those reported by Picot Groz

*et al*. (2014) (11) and by Bachelot *et al*. (2012) (56) for mussels collected from Portuguese and French coasts, respectively, where seasonal trends for these compounds were reported with the highest concentrations detected in the summer period, after the start of the bathing season. On the other hand, in this monitoring campaign no samples were collected during the summer period, only before (July) and after (October), making it difficult to compare.

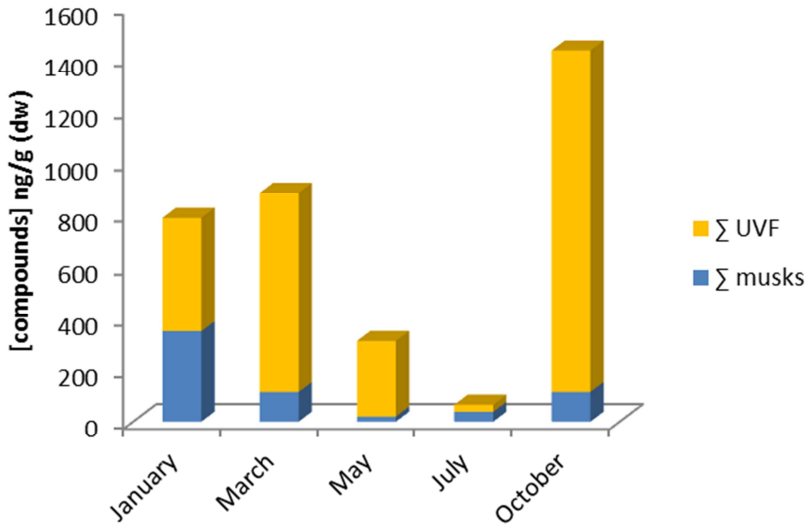



**Fig. 20** – Seasonal change of the total UV filters concentrations (ng/g dw) observed in mussels during the monitoring study conducted in 2015.

UV filters were the predominant contaminants between March and October, and musks reach higher levels in January, but still below UV filters (349.2 and 437.5 ng/g (dw), respectively).

# Chapter 4

## GENERAL CONCLUSIONS AND FUTURE TRENDS

The present work reports the improvement and further application to real samples of an already established QuEChERS-DLLME-GC/MS methodology for the simultaneous detection and quantification of seven musk fragrances (celestolide, galaxolide, tonalide, musk moskene, musk ketone, musk xylene and ethylene brassylate) and five UV filters (EHS, BP-3, 4-MBC, IMC and EHMC) in marine mussels (M. *galloprovincialis*). QuEChERS/DLLME extraction procedure proved to be a useful extraction method to selected PCPs, using low volume of organic solvents. Recoveries were higher than 72% for musks and 69% for UV filters. The analytical method allowed the determination of the target analytes at low concentrations in the order of few ng/g dw, from marine organisms exposed in coast water. The method was applied to wild mussel samples collected in seven different sites on the south coast of Portugal in five different periods during 2015. The occurrence of the target compounds varied depending on localization or on season. Two musk fragrances (musk ketone and ethylene brassylate) were not found in any samples. Two UV filters (EHS and EHMC) were detected in 73% of the samples, suggesting their ubiquitous contamination and widespread distribution. EHMC and BP-3 were detected at higher levels than EHS, 4-MBC and IMC, due probably to their wide presence in the formulation of several PCPs but also in food additives and detergents. Overall, higher levels were detected after the bathing season in October. Finally, this study revealed the occurrence and widespread contamination by emerging pollutants, such as synthetic musks and UV filters, in coastal waters of Portugal. However, little information on ecotoxicological implications of such chemicals is available. 4-MBC, BP-3 and EHMC routinely exceed the MAC established as environmental quality standards (EQS) of some compounds under the EU Water Framework Directive (above to 50 ng/g).

In view of the results obtained it is reasonable to suggest that some of the compounds here studied could be appropriate for inclusion in future coastal bivalve monitoring efforts based on their high concentrations or detection frequencies.

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